THE DEVELOPMENTAL GENETICS OF MOUSE EMBRYONAL STEM CELLS AND EMBRYONAL CARCINOMA CELLS

A Thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Methods for the selection of metabolic cooperation defective variant cell lines have been adapted to enable the isolation in three steps and four steps respectively of the cooperation defective variants 1P9 and 2P2[1] from the embryonal stem (ES) cell line B2B2TG5. Revertant cell lines restored in their ability to cooperate were isolated in one round of selection by the HAT "kiss-of-life" selection. Characterisation of these cell lines has shown that the cooperation-defective variants are both restricted in their in vitro differentiation capacity while the revertant derived from 1P9, designated 2H4, has its differentiation phenotype restored to a level comparable to that of the parent line. These results are interpreted as indicating that the metabolic cooperation deficiency and the restricted embryoid body differentiation phenotype of 1P9 are causally related. The revertant derived from 2P2[1], designated H19, remains severely restricted with regard to embryoid body differentiation, suggesting that in these lines a secondary event deleterious to differentiation, but unrelated to metabolic cooperation, has taken place.

In chapter 4 an investigation of the effects of mutagenesis upon the differentiation capacity of ES cell populations is reported. Three mutagenic agents were tested; the frame-shift mutagen ICR-191, the base substitution mutagen N-methyl-N'-nitrosoguanidine (MNNG),

and the deletion mutagen 1,2,7,8-diepoxyoctane (DEO). It was found that under the conditions employed, none of these elicited any detectable deleterious effects upon the differentiation capacity of the ES cell lines B2B2 and E14 (as assayed by embryoid body formation).

Chapter 5 reports strong inhibition of metabolic cooperation in B2B2TG5 by the aliphatic alcohols 1-heptanol and 1-octanol. Also presented is a preliminary investigation of the effect of 1-heptanol upon intercellular nucleotide transfer in the metabolic cooperation variant PTmr0, selected for resistance to the inhibition of metabolic cooperation by retinoic acid. These findings show that 1-heptanol is effective in blocking metabolic cooperation in this variant.

Chapter 5 describes a method for the bulk production of Buffalo rat liver (BRL) conditioned medium using microcarrier culture.

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Finally I would like to reserve a special mention for the many friends which I have made in Edinburgh, who have made my time here a pleasant one, and most especially my parents who have given me unstinting support and encouragement at all times.

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ABBREVIATIONS

AFP	Alphafetoprotein
BRL_{cm}	Buffalo Rat Liver cell conditioned medium
BUdR	5-Bromodeoxyuridine
cAMP	cyclic Adenosine mono-phosphate
CFDA	Carboxy fluorescein diacetate
CMß	Complete Medium supplemented with ß-mercaptoethanol
CRABP	Cellular Retinoic Acid Binding Protein
DAB	Diamino benzidine
dCK	deoxycytidine Kinase
DEO	1,2,7,8-Diepoxyoctane
DFMO	Difluoromethyl ornithine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC	Embryonal Carcinoma cell
ES	Embryonal Stem cell
fcs	foetal calf serum
HAT	Hypoxanthine, Aminopterin, Thymidine
HMBA	Hexamethylene bis-acetamide
HPRT	Hypoxanthine guanine phosphoribosyltransferase
ICM	Inner Cell Mass
IMP	Inosine monophosphate
LD_{50}	dose of a substance at which 50% of cells are killed
mec^+	metabolic cooperation competent
mec^-	metabolic cooperation defective
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mRNA	messenger RNA

ncs	new-born calf serum
NSS	Normal swine serum
ODCase	Ornithine decarboxylase
oua^s	ouabain sensitive
oua^r	ouabain resistant
PA	Plasminogen Activator
PAS	Periodic acid Schiff stain
PBSA	Phosphate buffered saline, Ca^{2+} and Mg^{2+}-free
PRPP	Phosphoribosyl pyrophosphate synthetase
RA	Retinoic acid
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBS	Tris.cl buffer pH 7.6
tG	6-thioguanine
tGMP	thioguanine monophosphate
TK	Thymidine Kinase
TPA	4β-phorbol 12-myristate 13-acetate
TVP	Trypsin/Versene dissolved in PBSA
L cell	Mouse fibroblast cell line (Earle, 1943). Metabolic cooperation defective (Pitts, 1971).

CHAPTER 1

INTRODUCTION TO THESIS

1.1 GENERAL INTRODUCTION

While it has long been suspected that gap junction mediated intercellular communication may be involved with developmental processes, very little admissible evidence has been presented to support this hypothesis. What little evidence exists is either circumstantial, pointing to the ubiquity of gap junctions within developing systems or, for example, to the fact that all variant embryonal carcinoma (EC) cell lines defective in gap junction-mediated intercellular communication isolated to date are also developmentally restricted (see section 1.18); or is subject to methodological criticism (for example the set of experiments described in section 1.16 involving the treatment of Xenopus oocytes and Hydra with antibodies raised against a putative gap junction protein).

The central aim of this thesis is to address the question of what role, if any, intercellular junctional communication plays in the development of the early mouse embryo. To this end, the *in vitro* differentiation of mouse embryonal stem cells (ES cells) has been employed as a model system. This study is concerned in particular with the cellular interactions involved in the process of embryoid body cavitation and related events which occur in ES cell-based model systems *in vitro*, and the relationship which this might bear to early events in mouse embryogenesis *in vivo*. Cell lines with altered

junctional communication phenotypes have been isolated and characterised, and the information gained from these variant lines is discussed with regard to several hypothetical models for cellular determination.

In this chapter, the normal development of the early mouse embryo is discussed, and the status of EC/ES cells as a model system reviewed. Also reviewed is the current state of knowledge regarding gap junctions, with special reference to their potential involvement in development.

1.2 THE EARLY DEVELOPMENT OF THE MOUSE

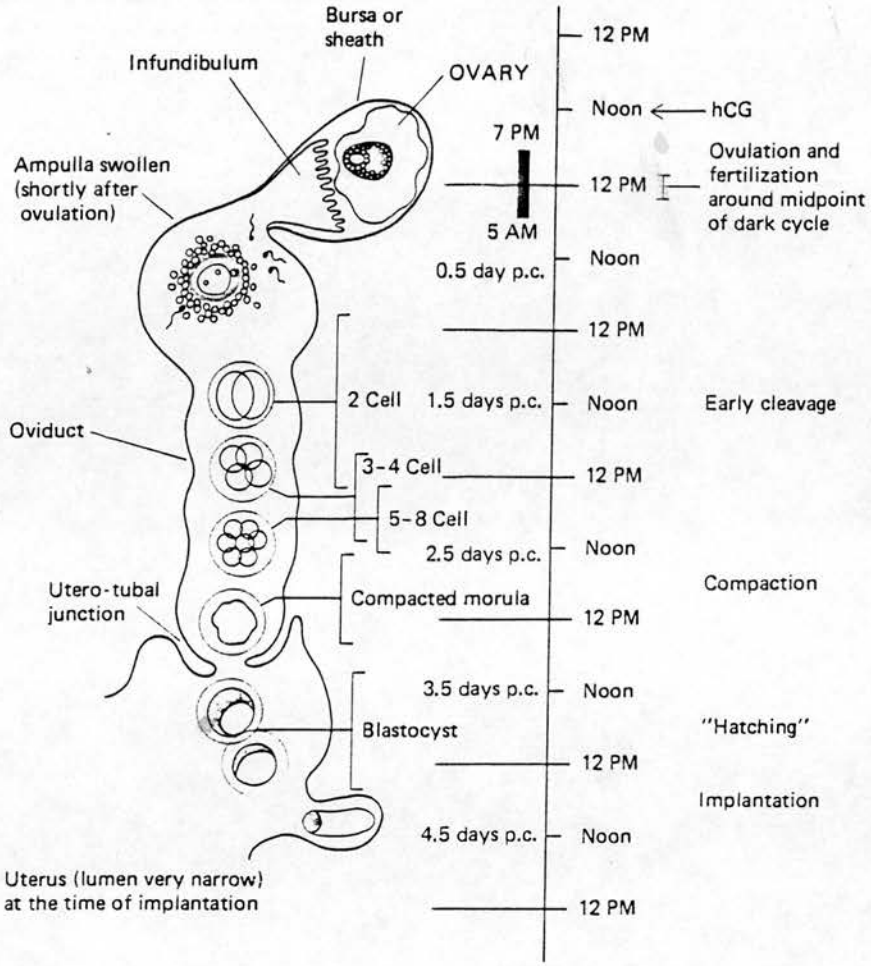
Unless otherwise stated, the information contained within this section has been derived from Hogan, Costantini and Lacy (1986). The early events in mouse embryo development are represented schematically in figure 1.1.

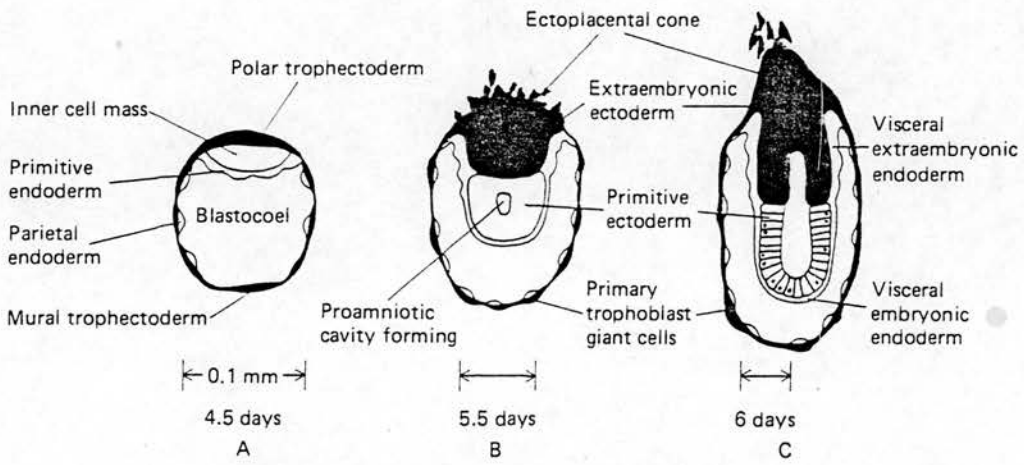
The embryonic development of the mouse begins by fertilisation of the eggs (typically 8-12 per ovulation) by the sperm in the uppermost loop of the oviduct (the Ampulla Tubae). Some 58 x 10^6 sperm are released into the female reproductive tract per ejaculation. Some of these reach the ampulla within 5 minutes, but they are not competent for fertilisation for about 1 hour. The mechanism of this maturation process, known as capacitation, is unknown. In order to reach the surface of the egg, the sperm must first penetrate the cumulus

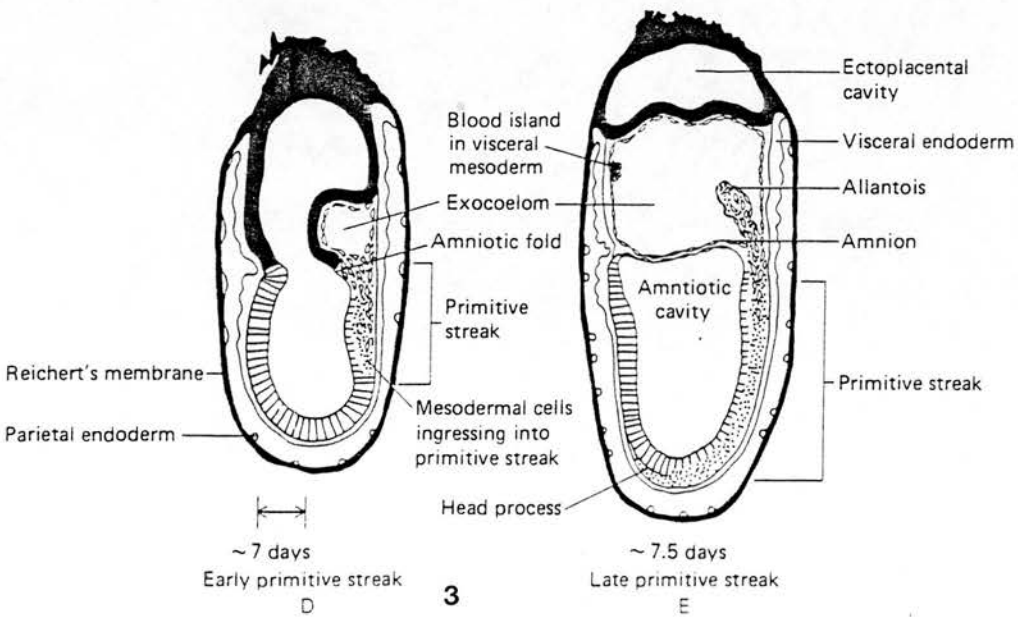
FIGURE 1.1

The early development of the mouse embryo from ovulation to 7.5 days.

Adapted from Hogan, Costantini and Lacy (1986).


Bursa or sheath
Infundibulum
OVARY
Ampulla swollen (shortly after ovulation)
Oviduct
Utero-tubal junction
Uterus (lumen very narrow) at the time of implantation
12 PM
Noon
hCG
7 PM
12 PM
5 AM
Ovulation and fertilization around midpoint of dark cycle
Noon
0.5 day p.c.
12 PM
2 Cell
1.5 days p.c.
Noon
Early cleavage
3-4 Cell
12 PM
5-8 Cell
2.5 days p.c.
Noon
Compaction
Compacted morula
12 PM
3.5 days p.c.
Noon
Blastocyst
"Hatching"
12 PM
Implantation
4.5 days p.c.
Noon
12 PM


Polar trophectoderm
Inner cell mass
Primitive endoderm
Blastocoel
Parietal endoderm
Mural trophectoderm
0.1 mm
4.5 days
A
Ectoplacental cone
Extraembryonic ectoderm
Primitive ectoderm
Proamniotic cavity forming
Primary trophoblast giant cells
5.5 days
B
Visceral extraembryonic endoderm
Visceral embryonic endoderm
6 days
C


Blood island in visceral mesoderm
Exocoelom
Amniotic fold
Primitive streak
Reichert's membrane
Parietal endoderm
Mesodermal cells ingressing into primitive streak
~7 days
Early primitive streak
D
Ectoplacental cavity
Visceral endoderm
Allantois
Amnion
Amntiotic cavity
Primitive streak
Head process
~7.5 days
Late primitive streak
E

mass and the zona pellucida. This is achieved by the release of various proteolytic enzymes from a vacuole-like structure in the head of the sperm known as the acrosome. Once the head of the sperm has fused with the egg membrane a cascade of reactions are set in motion. One of the earliest events is a change in the egg surface inhibiting the fusion of additional sperm. During fertilisation, the head, midpiece, and a large part of the tail of the sperm are all incorporated into the egg cytoplasm. The midpiece contributes paternal centrioles and mitochondria to the egg (although the mitochondria are enormously outnumbered by those of the egg). Over the next 20 hours or so, the residual follicle cells which surrounded and nourished the ovum are lost as the egg migrates down into the second loop of the oviduct. During this time, the haploid male and female pronuclei approach one another and DNA replication takes place. The nuclear membranes then break down and the chromosomes assemble on the spindle. The first cleavage division takes place soon after. Over the next 48 hours the cleavage stage embryo undergoes a further three rounds of division to generate a morula composed of 16 individual blastomeres. These are roughly spherical and relatively autonomous until compaction (Adamson and Gardner, 1979), when the blastomeres flatten and increase their contact with each other. Tight junctions form between outside cells, effectively sealing off the inside of the ball of cells from the outside environment. These changes are accompanied by extensive reorganisation of the

cytoskeleton and redistribution of mitochondria and microvilli (Adamson and Gardner, 1979; Johnson, 1981).

The establishment of disparate microenvironments between the inside and outside of the developing embryo leads to a progressive divergence of the cells resulting in the generation of two distinct lineages: the trophectoderm (TE) and the inner cell mass (ICM) (reviewed by Johnson, 1981; Gardner, 1983).

By the fourth day of development, the embryo is in the uterus and is composed of approximately 35 cells. At this stage a large eccentrically placed lumen, known as the blastocoel, develops (Adamson and Gardner, 1979). The inner cell mass cells become localised as a compact cluster of cuboidal cells at one pole of the developing blastocyst. The fully expanded blastocyst contains about 64 cells, of which about 20 are in the ICM.

During the 5th day of development the blastocyst "hatches" from the zona pellucida and implants in the uterine wall. As this takes place the ICM undergoes differentiation into primitive ectoderm (or epiblast) and a layer of primitive endoderm cells form at the interface between the ICM and the blastocyst cavity (Enders *et al.*, 1978). This development may be initiated by an inside-outside difference as with the earlier bidirectional differentiation of the morula (Gardner, 1983). Primitive ectoderm cells transiently become less

closely associated with one another during delamination of the endoderm (Enders *et al.*, 1978). The primitive ectoderm is destined to give rise to the foetus and to the amniotic ectoderm and extraembryonic mesoderm, while the primitive endoderm will give rise to the extraembryonic visceral and parietal endoderm (Gardner and Papaioannou, 1975; Gardner and Rossant, 1979; Gardner, 1983).

1.3 EARLY POST-IMPLANTATION DEVELOPMENT

Post-implantation, the trophectoderm undergoes differentiation into two distinct lineages: the mural trophectoderm which develops from the portion of trophectoderm surrounding the blastocoelic cavity, and the polar trophectoderm derived from that portion of trophectoderm which comes into contact with the inner cell mass. Mural trophectoderm cells cease division and become large and polyploid (primary trophoblastic giant cells). They can contain up to 1000 times the haploid amount of DNA (Ilgren, 1981). The mural trophectoderm invades the uterine epithelium and its underlying basal lamina and then penetrates the uterine stroma. Polar trophectoderm remains diploid and continues to proliferate rapidly. First, some cells migrate around the embryo, replacing the primary mural trophoblastic giant cells and themselves becoming polyploid. Second, a finger-like projection of polar

trophectoderm called the ectoplacental cone penetrates the blastocoel cavity, forming the extraembryonic ectoderm of the egg cylinder and pushing the ICM derivatives ahead of itself. The epiblast simultaneously proliferates and bulges into the blastocoelic cavity to form the embryonic portion of the egg cylinder by day six.

As the above changes take place, the primary endoderm layer also undergoes differentiation into two morphologically, biochemically and topographically distinct cell populations. The initial event taking place shortly after implantation is the differentiation of primary endoderm into parietal endoderm which then proliferates and migrates away from the epiblast to line the blastocoelic cavity. Following this, a second wave of primary endoderm delamination precedes differentiation into visceral endoderm. This remains in contact with the epiblast.

The blastocoelic cavity is now surrounded by an outer layer of trophoblast giant cells lined with parietal endoderm, and at this stage is termed the parietal yolk sac. The parietal endoderm cells assume a fibroblastic morphology and form limited cell contacts with the overlying trophoblastic giant cells, as they rapidly secrete a thick basement membrane known as Reichert's membrane (Theiler, 1972; Adamson and Ayers, 1979). This is largely composed of type IV collagen (Adamson and

Ayers, 1979), laminin (Timpl and Rhode, 1979; Hogan, 1980; Hogan *et al.*, 1980; Dziadek and Timpl, 1985), heparan sulphate and other sulphated glycoproteins (Hogan *et al.*, 1982). Little is known about the function of Reichert's membrane, although it has been postulated that it may act as a crude filter for substrates passing from the maternal to the foetal environment (Jollie, 1968). Parietal endoderm cells also secrete the tissue activator form of plasminogen activator (Strickland *et al.*, 1976; Marotti *et al.*, 1982), as distinct from the urokinase form produced by the mature visceral endoderm (Marotti et al, 1982).

The visceral endoderm matures into an epithelium consisting of a single layer of closely apposed cuboidal cells joined by apical junctional complexes (Enders *et al.*, 1978). Although residing upon thin deposits of extracellular matrix similar in composition to Reichert's membrane (Wartiovaara *et al.*, 1979; Leivo *et al.*, 1980), they remain in junctional contact with the epiblast by virtue of cytoplasmic projections from the latter which interrupt the basal lamina (Gardner, 1983). The visceral endoderm can endocytose and degrade proteins and pass on the free amino acids generated to the embryo (Gardner, 1983), suggesting that this cell layer may play a key role in the nutrition of the embryo. Together with the visceral mesoderm it forms the visceral yolk sac which envelops the growing embryo.

Visceral endoderm may be immunocytochemically visualised in the embryo from the sixth day post-fertilisation by virtue of the fact that it is the unique source of alpha-foetoprotein (AFP) (Dziadek and Adamson, 1978), transferrin (Adamson, 1982) and endogenous peroxidase (Balakier, 1984) at this stage. However, transferrin is also synthesised by primitive ectoderm from day 7 and AFP is not synthesised by the extraembryonic portion of the visceral endoderm as its production is suppressed by the extraembryonic ectoderm (Dziadek, 1978). Also at later stages of development the surrounding tissues may adsorb AFP, although this problem can be overcome by in situ hybridisation to mRNA localising the sites of synthesis of AFP (Kurkinen *et al.*, 1983; Dziadek and Andrews, 1983).

At about the same time as visceral endoderm maturation and implantation, a small cavity begins to form within the inner core of primitive ectoderm cells (day 5.5). This appears to involve some cell death, as cellular debris is frequently observed within the cavity. This proamniotic cavity gradually becomes organised into a simple epithelium surrounding the central lumen. The primitive ectoderm proliferates rapidly during this period so that in the day between cavity initiation and commencement of gastrulation the cell population expands from approximately 120 cells to about 660 cells (Snow, 1977, 1978).

Gastrulation begins at about 6.5 days post-coitum when there are about 1000 cells in the primitive ectoderm. The proamniotic cavity expands and cells delaminate from the epithelial layer of the primitive ectoderm and accumulate as a layer of individual mesoderm cells between the primitive ectoderm and visceral endoderm. The region of mesoderm differentiation is known as the primitive streak, and its appearance establishes the anterior-posterior axis of the embryo. At about the same time as primitive streak formation, the amniotic folds begin to cut off the embryonic region of the proamniotic cavity from the extraembryonic region, forming two cavities termed the amniotic cavity and the ectoplacental cavity respectively. These cavities are separated by the simultaneous encroachment of a third cavity, the exocoelom, arising from expansion of the posterior end of the primitive streak, so that by 7.5 days post-coitum the embryo has developed from a single cell into a complex, highly ordered, multi-layered structure.

The germ-line becomes apparent in histological sections about 9 days post-coitum, within the epithelium of the hind-gut. These then migrate into a structure called the genital ridge, which subsequently differentiates to give rise to the gonads. Sexual differentiation becomes apparent on about the 12th day of gestation (Theiler, 1972).

1.4 EMBRYONAL CARCINOMA CELLS

Embryonal carcinoma (EC) cells are the rapidly dividing cells which give rise to teratocarcinomas. The latter are malignant tumours, usually of the gonads, characterised by the presence of a wide variety of differentiated cell types derived from the EC stem cell population (Stevens, 1983). Typically, teratocarcinomas contain derivatives from all three germ layers; ectoderm, mesoderm and endoderm, arranged in a chaotic fashion. Extra-embryonic tissues, such as yolk-sac, are also commonly found. Teratocarcinomas in which all EC cells have differentiated are benign, and are known as teratomas (although this term is frequently also applied to the malignant form).

EC cells can be readily isolated from teratocarcinomas and propagated *in vitro*. Transplantation of murine EC cells back into mice gives rise to further teratocarcinomas. *In vitro*, EC cells can be induced to differentiate either in monolayers, or to form structures known as embryoid bodies which are analogous in some respects to the early post-implantation embryo (see section 1.3).

EC cells resemble early embryonic (inner cell mass/primitive ectoderm) cells in that they are "pluripotent", i.e. they can differentiate into a wide variety of cell types. Also they are morphologically and

biochemically similar to early embryonic cells, and in some instances have proved capable of contributing to chimaeric animals (Mintz and Ilmensee, 1975; Stewart and Mintz, 1981; Fujii and Martin, 1983). These properties, coupled with the ease with which they can now be manipulated *in vitro*, makes them an excellent model system for studies of early embryogenesis.

It has been suggested that EC cells are normal embryonic cells which are rendered malignant by virtue of an abnormal microenvironment. This hypothesis is strongly supported by the recent isolation of EC-like cells directly from implantation-stage mouse embryos (Evans and Kaufman, 1981; Martin, 1981; Axelrod, 1984; Hooper *et al*, 1987; Suemori and Nakatsuji, 1987). These are known as embryonic stem cells (EK or ES cells, see section 1.7).

1.5 TERATOMAS AND TERATOCARCINOMAS

While teratomas are rare in nature, they do occur at high frequency in certain inbred strains of mice. In 1954 Stevens and Little found that about 1% of male mice of the inbred strain 129 had testicular teratomas. These start as small nests of cells in the genital ridge at about the 15th day of gestation, and are thought to derive from the primordial germ cells (Stevens, 1983). These start to develop at about 12.5 days, and grafting of genital ridges from foetuses at this stage to adult

testes produces teratomas at high frequency. If, however, the graft is performed from 12 day old foetuses or earlier (before the primordial germ cells have migrated to the genital ridge), no teratomas result. Also genital ridge grafts from embryos homozygous for the Sl allele, which prevents primordial germ cell formation, fail to produce teratomas. These observations support the view that in males teratomas derive from the primordial germ cells.

The situation in female mice is somewhat different. Teratomas have not been derived from female primordial germ cells, probably because on the 13th day of gestation they enter meiosis (but arrest before completing it) whereas in the male they do not undergo meiosis until after birth (Martin, 1981). Spontaneous ovarian teratomas occur in about 50% of strain LT/Sv female mice. They derive from eggs which begin to develop parthenogenetically within the ovary, and undergo apparently normal cleavage and blastocyst formation. At the egg cylinder stage the embryos become disorganised and teratomatous. Unlike testicular teratomas, ovarian teratomas are frequently associated with trophoblastic giant cells, probably as a consequence of their development via blastocysts, which possess a trophectoderm component.

Teratomas can also be produced experimentally from both male and female mice by transplantation of 1-7 day old

embryos to ectopic sites provided that the foetal component of the embryo is included in the graft (Solter and Damjanov, 1973; Stevens, 1983). After 7 days all the embryonic cells have become determined and differentiate, and may give rise to benign teratomas but not to transplantable teratocarcinomas. This, along with the fact that EC-like cells can be isolated directly from blastocyst stage embryos, supports the case for an embryo cell origin for ovarian teratomas.

Teratomas are usually benign, the EC cells having differentiated into cell types which stop proliferating after a few generations. Malignant teratocarcinomas still contain undifferentiated EC cells and will form new teratocarcinomas if transplanted. As the passage number increases they tend either to differentiate and become benign, or to lose their potency so that the variety of cell types present within tumours becomes somewhat restricted. If transplantable teratocarcinoma cells are injected intraperitoneally they develop into small rounded aggregates which superficially resemble the embryonic portion of the egg cylinder and for this reason are called embryoid bodies. These float freely in the ascitic fluid. Embryoid bodies generally consist of a core of EC cells surrounded by an outer layer of endoderm. Some embryoid bodies develop large cystic structures and differentiate extensively internally, resembling somewhat later embryonic stages (Martin and Evans, 1975a,b,c). This process can be duplicated *in*

vitro and provides a useful assay for the developmental potential of EC/ES cells (Uno, 1982).

To summarise, teratocarcinomas/teratomas can be derived from male primordial germ cells, female parthenogenetic egg cells and embryonic inner cell mass/primitive ectoderm cells. Research has shown that all these cell types are similar with respect to their developmental capacity, and also morphologically, biochemically and ultrastructurally. In view of their ability to contribute in a non-malignant way to chimaeric animals, the malignancy of such cells in teratocarcinomas is a function of the abnormal microenvironment within which they find themselves (i.e. in the absence of the normal embryonic environment), or in the case of parthenogenetic eggs, of the lack of the male gametic contribution.

1.6 ORIGINS OF EMBRYONAL CARCINOMA CELLS

Permanent EC cell lines have been established from teratocarcinomas or embryoid bodies derived by all of the methods described above. Also, similar cell lines have been directly obtained from the inner cell mass of blastocyst stage embryos (EK or ES cells). The latter are described in section 1.7.

EC cells are relatively small (12-14μm), diploid or nearly so, and phenotypically similar to undifferentiated

embryonic stem cells. The nucleus is large and contains 2 or 3 prominent nucleoli, and euchromatin predominates over heterochromatin. The cytoplasm typically contains few mitochondria and many free ribosomes. Also there are small quantities of rough endoplasmic reticulum and a primitive Golgi body. This morphology is typical of all EC cells, whatever their derivation, and is also characteristic of primordial germ cells and embryonic ectoderm.

The majority of EC lines require the presence of a layer of mitotically arrested fibroblastic cells (known as feeder cells) for proliferation and maintainance in an undifferentiated state *in vitro*. Removal of feeder cells from the culture environment causes such "feeder-dependent" cells to differentiate. Some EC cell lines are feeder independent, and will not spontaneously differentiate in the absence of feeders. Such lines may be induced to differentiate with limited potency by the presence of a chemical inducer such as retinoic acid, or by plating at high densities in an aggregation promoting medium (see section 1.8 and 1.9), but are generally somewhat abnormal and are therefore more limited in their uses than the feeder-dependent lines.

In the past, while feeder-dependant lines have obviously been more attractive than feeder-independant lines for developmental studies, the presence of feeder cells has presented a number of problems. For example, biochemical

studies have been virtually impossible and genetic manipulations of cells (e.g. selection of HPRT- mutants) have been grossly hampered due to the phenomenon of metabolic cooperation with the feeders (see sections 1.11 and 1.18). Recently though it has been found that the feeder effect can be partially reproduced by feeding cells with medium previously "conditioned" by incubation for a period of time upon a layer of feeder cells (Smith and Hooper, 1983), and subsequently that medium conditioned by Buffalo rat liver (BRL) cells completely reproduces the feeder effect (Smith and Hooper, 1987). This represents a major advance in stem cell biology as it enables workers to maintain and manipulate completely homogeneous populations of EC and ES cells in culture for the first time.

1.7 ISOLATION OF PLURIPOTENT STEM CELLS (ES CELLS) FROM PREIMPLANTATION STAGE MOUSE EMBRYOS

As mentioned in the previous sections, EC-like cells have recently been successfully derived from preimplantation mouse blastocysts. These cells are termed EK or ES cells and are totipotent embryonic stem cells maintained in an undifferentiated state in mass culture. This represents a significant step in mammalian developmental biology, as it has long been recognised that while EC cells are analogous to the stem cells of the early embryo, the vast majority of EC lines are abnormal in some respects and

thus their uses as a model system for embryogenesis are somewhat limited.

Also, another exciting possibility now presents itself; that of deriving pluripotent stem cell lines from species other than mouse, particularly the larger animals from which stem cells could not be derived in the past simply because of the prohibitive expense and difficulty of maintaining sufficient stock to obtain the occasional teratocarcinoma. Towards this goal, Handyside *et al* (1987) have recently attempted the isolation of ES cells from sheep embryos.

Embryonic stem cells were first isolated by Evans and Kaufman (1981), who called their cell lines EK cells. Initially it was thought that the low number of cells in the ICM of the normal blastocyst was responsible for early failures to isolate the ICM cells, so their protocol employed a combination of ovariectomy and Depo Provera injection, which induces diapause and thus causes delayed implantation of the blastocyst, resulting in an increase in the size and cell number of the ICM. Using this method they were able to sucessfully isolate lines with a karyotype of 40XY which, by trypsin banding, appeared to be normal as compared to other somatic mouse cells. Martin (1981) used medium conditioned by EC cells (PSA4) in which to isolate embryonic stem cell lines. Subsequently, it has been demonstrated that neither of these procedures are necessary (Robertson *et al*., 1983;

Axelrod, 1984). Euploid pluripotent ES cell lines can be derived from individual pre-implantation embryos by culture of intact blastocysts or immunosurgically isolated ICMs in microlitre volumes of serum-containing medium upon a fibroblast feeder layer (Axelrod, 1984). It is thought that the initial use of small volumes of medium enables the stem cells to condition their own medium sufficiently to support their growth into large enough numbers to allow the formation of a permanent line. Feeder cells are necessary, probably to inhibit differentiation, but a recent report which claims that STO feeders cause ES lines to become tetraploid whereas primary fibroblasts do not (Suemori and Nakatsuji, 1987) indicates that caution must be exercised in choosing a suitable feeder layer. However, in view of the fact that STO feeders have been employed sucessfully in stem cell isolation by the majority of other workers, it would seem likely that the batch used for this particular set of experiments was in some way abnormal.

ES cell lines have now been established from a variety of inbred and outbred strains of mice. Kaufman *et al* (1983) have isolated homozygous diploid lines of parthenogenetic origin. Also, it has proved possible to isolate ES cell lines bearing homozygous lethal developmental mutations. Magnuson *et al* (1982), have isolated ES cell lines homozygous for the t^{w5} mutation of the t complex. Interestingly these cells differentiate extensively *in vitro* and in tumours.

ES cells and EC cells are indistinguishable on the basis of morphology, cell surface antigen expression, patterns of protein synthesis and differentiative behaviour *in vitro* and *in vivo* (Evans and Kaufman, 1983), confirming the analogy between EC cells and epiblast cells of the early post-implantation embryo and indicating that EC cells are a valid model system for the study of the process of embryonic development. However, in view of the advantages of ES cells, i.e. their isolation from normal embryos, variety of genotypes, retention of euploidy and contribution to the germ-line in chimaeras, it seems likely that for developmental biology work they will largely supersede EC cells as a model system.

1.8 DEVELOPMENTAL POTENTIAL OF EC AND ES CELLS

EC and ES cells are capable of differentiating into a wide variety of cell types, representing all three embryonic germ layers and also in some cases extra-embryonic tissues such as yolk sac and trophectoderm. This has been observed both *in vivo* in the form of differentiating teratocarcinomas and embryoid bodies, and *in vitro* in the form of embryoid bodies both in suspension and in outgrowth form (Martin, 1975). Differentiation of EC and ES cells can also be induced *in vitro* by plating cells at low density upon a gelatin-coated substratum in the absence of feeders or of

suitable conditioned medium.

Teratocarcinomas may be produced by subcutaneous injection of 10^4 to 10^6 EC cells into syngeneic mice, or by grafting small populations of EC cells under the kidney capsules of syngeneic mice (Rayner and Graham, 1982). Stevens (1959) first described embryoid bodies in teratocarcinomas of strain 129 mice, and subsequently it was discovered that intraperitoneal injection of disaggregated 129 teratocarcinomas led to the formation of large numbers of embryoid bodies freely suspended in the ascitic fluid (Pierce and Dixon, 1959). They are composed of EC cells arranged in clumps surrounded by an envelope of endodermal cells. The inner cells of such embryoid bodies may remain undetermined for several years, while still retaining their multipotentiality. Plating the embryoid bodies obtained from the ascites cavity of a mouse in suspension culture results in further differentiation, giving rise to yolk sac membranes, mesoderm and blood islands (Jacob, 1977). When single embryoid bodies are grafted to the testis (Stevens, 1960) or subcutaneously (Pierce and Dixon, 1959), they give rise to teratocarcinomas composed of various tissues.

In vitro, embryoid bodies may be produced by plating EC cells at high density under conditions which encourage aggregation, then detaching the aggregates and seeding them over a substrate to which they cannot attach

(bacteriological dishes, for example) (Martin and Evans, 1975a). Alternatively, EC cells can be induced to aggregate by seeding at low density on feeders and transferring individual colonies intact into bacteriological dishes, or by directly passaging poorly dissociated cultures directly into bacteriological dishes. The aggregates of pluripotent cell lines, including PSA4, round up to become clumps of EC cells surrounded by an endodermal layer, and subsequently differentiate further, the endodermal layer becoming composed of both parietal and visceral endoderm, and the inner core developing a cavity typically lined with columnar epithelium, and closely associated with various other differentiated tissues. Nullipotent EC lines, such as F9 and PC13, fail to differentiate under these conditions, and form homogeneous aggregates of embryonal carcinoma both *in vitro* and *in vivo* (Martin and Evans, 1975a; Martin *et al.*, 1977).

Embryoid body formation has a number of features in common with early embryonic development. The formation of the endoderm layer resembles the delamination of endoderm on the free surface of the ICM of the implantation stage mouse embryo. Isolated ICMs have been found to form endoderm over their entire outer surface (Rossant, 1975; Gardner and Papaiannou, 1975). In contrast, primitive ectoderm cores isolated from implantation stage mouse blastocysts do not regenerate endoderm (Gardner, 1983). The evidence thus suggests that EC cells are

developmentally equivalent to ICM cells. This is at odds with comparisons between the protein synthesis patterns of EC cells, ICM and primitive ectoderm, which indicate that EC cells more closely resemble primitive ectoderm than ICM (Dewey *et al.*, 1978; Lovell-Badge and Evans, 1980; Evans *et al.*, 1979). It may be that the development of intact primitive ectoderm cores is regulated by epigenetic factors rather than genetically restricted (Gardner, 1983).

The development of cavitated embryoid bodies is in some ways analogous to differentiation of the egg cylinder in the early post-implantation embryo, although there are differences in detail (Boyd *et al.*, 1984). Between the fourth and sixth day of suspension culture one or more eccentrically-orientated cavities appear in association with focal necrosis (Martin *et al.*, 1977; Boyd *et al.*, 1984), and the surrounding EC cells differentiate into a layer of columnar epithelium resembling mature embryonic ectoderm, which lines the inner wall of the cavity. Cultured ICMs undergo a similar process of cavitation, although in this case a large proportion develop into paired cysts (Wiley *et al.*, 1978). Cavitation appears to be a neccessary precursor to further differentiation as embryoid bodies which fail to cavitate undergo no further differentiation whilst maintained in suspension (Martin *et al.*, 1977; Uno, 1982). If such non-cavitating embryoid bodies are plated onto a tissue culture surface, outgrowths composed of a wide variety of differentiated

cell types emerge after a short period of time (Evans and Martin, 1975; Martin and Evans, 1975a). This may be accounted for by the proposal of Evans and Martin (1975) that the cavity may provide a two-dimensional surface for the positional organisation necessary for further cell determination.

Cavitation is thought to be dependant upon the presence of an outer layer of endoderm. Martin *et al* (1977) observed that embryoid bodies of the cell line SCC-S2, which fail to cavitate, develop parietal endoderm but only traces of visceral endoderm, suggesting that the presence of visceral endoderm may be necessary for cavitation. The data of Smith *et al* (1986) based upon analyses of embryoid bodies derived from the EC cell line PSA4TG12 and its clonal derivatives, indicate that cavitation can proceed in the presence of either category of endoderm. However, a more detailed analysis by Fisher (1987) of cavitated embryoid bodies derived from PSA4TG12 and the ES line E14 has indicated that cavitated embryoid bodies devoid of alpha-foetoprotein positive staining cells (indicative of the presence of visceral endoderm) contain a third cell type apparently of endodermal origin, which is as yet unidentified but appears to be ultrastructurally intermediate between parietal and visceral endoderm and may be a precursor of mature visceral endoderm.

The differentiated cell types in cavitated embryoid

bodies are typically disorganised and the trilaminar arrangement of ectoderm, mesoderm and endoderm as seen in the primitive yolk sac is rarely observed in embryoid bodies. A similar lack of organisation is seen in cultured ICMs (Wiley *et al.*, 1978), indicating that it may arise as a consequence of the *in vitro* environment rather than from some abnormality of the EC cells.

The most striking demonstration of stem cell pluripotency is chimaera formation, either by injection of stem cells into the blastocyst of the preimplantation mouse embryo or by aggregation with morula embryos *in vitro* and then placing the aggregates back into the uterus of another animal for gestation (for review see Bradley and Robertson, 1986). In many cases, the progeny obtained following such manipulations have been shown to contain a wide range of apparently normal tissues derived in whole or in part from the EC or ES cells used (Mintz and Illmensee, 1975; Rossant and MacBurney, 1983; Bradley and Robertson, 1986). In general, EC lines are less efficient than ES lines with regard to chimaera formation, and abnormal progeny, or progeny containing EC cell-derived teratocarcinomas, are common (reviewed by Papaioannou and Rossant, 1983). The frequency of abnormalities and tumours is much reduced when ES lines are employed (Bradley and Robertson, 1986).

It has recently proved possible for genetically altered ES cells to be represented in the germ-line of chimaeric

mice and pure-breeding HPRT$^-$ (Lesch-Nyhan syndrome) mice have now been successfully bred from such chimaeras (Hooper *et al.*, 1987; Kuehn *et al.*, 1987). Further application of this technique for the creation of other animal models for human genetic disease is an exciting prospect.

1.9 CHEMICAL INDUCTION OF DIFFERENTIATION

Many nullipotent EC cell lines which fail to differentiate in any of the systems discussed above (or do so with very limited potency) can be induced to differentiate with chemical inducers. The list of such inducers is long, and constantly being added to, and consequently I shall devote the bulk of this section to a brief overview of one of the most potent, and certainly the most exhaustively investigated, inducers of EC cell differentiation; retinoic acid. Upon treatment with a differentiation inducer, EC cells generally differentiate into a limited variety of cell types which is dependent upon the culture environment, the cell line, the chemical used, and in some cases the concentration of the chemical. For example, PCC4aza1 cells differentiate to an epithelial morphology in monolayer and to a fibroblastic morphology in suspension in response to hexamethylene bisacetamide (HMBA), polybrene and N,N-dimethylacetamide (Speers *et al.*, 1979). Retinoic acid treatment of the EC line O1A1 induces differentiation into mainly cardiac

muscle at 10^{-9}M, skeletal muscle at 10^{-8}M and neurons and astroglia appear at concentrations between 10^{-7} and 10^{-5}M (Edwards and MacBurney, 1983). The latter observation is a particularly interesting one, as it suggests that a simple concentration gradient of a single chemical inducer could be responsible for instigating diverse developmental programmes across a field of cells in a developing system.

The action of retinoids is very complex and they exhibit a plethora of effects upon cellular functions and developmental processes. For example, as well as inducing differentiation in EC/ES cell lines, retinoic acid interferes with pattern formation in chick and axolotl limb formation (Maden, 1982); inhibits both the differentiation and proliferation of human bone marrow cells (Bradley *et al.*, 1983); induces the switching of cellular glycolipids from the globo-series to the lacto- and ganglio-series in TERA-2 derived human EC cells (a change which naturally occurs during embryo development) (Fenderson *et al.*, 1987); inhibits the synthesis of ornithine decarboxylase (ODCase) (the first enzyme in the pathway to polyamines) in a variety of cell types and suppresses its induction by growth factors and tumour promoters (Russel and Frasier-Scott, 1983; Jetten, 1984; Jetten and Shirley, 1985), thereby reducing the level of intercellular polyamines (Schindler *et al*, 1985); inhibits proliferation of certain tumours and antagonises tumour promoters such as TPA (for review see Schindler,

1986) and inhibits gap junction mediated intercellular communication at high concentrations (Pitts *et al.*, 1986). This multiplicity of effects has greatly complicated analyses of the mode of action of retinoic acid as applied to any single property, and it is likely that many of the observed effects of retinoic acid arise simultaneously as a consequence of some common pathway. For example, ODCase performs a key role in polyamine synthesis and so its inhibition will reduce the level of polyamines. There is mounting evidence for a role of polyamine levels in differentiation in various systems and significantly, specific inhibition of ODCase by the ornithine analog α-diflouromethylornithine (DFMO) results in the majority of cases in identical phenotypic changes to those brought about by retinoic acid treatment. A good example of this is the antagonistic effect of both retinoic acid and DFMO upon TPA-induced tumour promotion. It is known that TPA stimulates the production of ODCase and raises the intercellular level of polyamines, and it has been suggested that tumour promotion in this case may arise as a direct consequence of this activity (for review see Schindler, 1986).

It is likely that the cellular retinoic acid binding protein (CRABP) is an essential component in the mechanism of retinoic acid-induced differentiation as a number of studies have shown that differentiation defective EC cell lines lacking CRABP activity fail to respond to retinoic acid (Schindler *et al.*, 1981; McCue

et al., 1983; Wang and Gudas, 1984). Complementation analyses by cell fusion between various differentiation-defective variants have illustrated that all hybrids capable of responding to retinoic acid have detectable levels of CRABP activity (McCue *et al.*, 1984a), and McCue *et al* (1984b) have demonstrated that a CRABP-deficient EC cell mutant can differentiate in response to retinoic acid if also exposed to sodium butyrate, an agent that restores production of CRABP in these cells.

There is mounting evidence that retinoid-binding proteins may deliver exogenously added retinoids to nuclear sites, and that these nuclear sites possess relatively high affinities for the retinoids. This would suggest that retinoids might modulate gene expression by interaction with the genome, either directly or, as has been proposed by Chytil and Ong (1979), via their receptor proteins in a manner analogous to the action of steroid hormones and their receptor proteins. For an extensive review on this subject see Sherman (1986).

1.10 METHODS FOR THE DETECTION AND QUANTIFICATION OF DIFFERENTIATION

There are several widely employed tests for differentiation. Most commonplace is observation of cell morphology under the light microscope. However, although it is straightforward to detect the presence of

differentiated cell types by this method, problems arise with complex developmental systems such as EC/ES cell differentiation where a wide variety of cell types may be present, making quantitative assessment of the extent of differentiation a difficult task. Therefore in order to complement morphological examination, a number of cell-lineage specific markers are commonly employed. An antibody to the lineage-specific protein alpha-foetoprotein (see section 1.3), expressed mainly by visceral endoderm and liver cells (Dziadek, 1978; Dziadek and Adamson, 1978), is an example of this category of marker. Non-specific markers which simply indicate the occurrence of differentiation are also available, for example plasminogen activator (PA) is produced in large amounts by a majority of differentiated cell types but not in detectable amounts by undifferentiated cell types such as EC cells (Sherman *et al.*, 1976; Linney and Levinson, 1977; Strickland and Mahdavi, 1978). However, there are some reservations about the reliability of the PA assay as it is known that certain differentiated cell types fail to produce PA, and that PA production can be induced by exposure to UV light in cells with a DNA repair defect (Miskin and Ben-Ishai, 1981), suggesting that it may be instigated by unrepaired DNA damage. Thus, caution must be exercised when interpreting the results of experiments using PA as evidence for differentiation.

1.11 METABOLIC COOPERATION

Metabolic cooperation is defined as the ability of cells to exchange molecules through permeable junctions formed at the sites of cell contact (Hooper and Subak-Sharpe, 1981). The term was first coined by Subak-Sharpe, Burk and Pitts (1966) to describe the ability of variant cells in culture to incorporate a nucleic acid precursor when in contact with wild type cells, but not when on their own. This is distinct from cross feeding, where material is transferred from one cell to another via the extracellular fluid (Hooper and Subak-Sharpe, 1981). The permeable channel responsible for metabolic cooperation is the gap junction (section 1.13).

1.12 THE DETECTION AND QUANTIFICATION OF METABOLIC COOPERATION

Junctional communication is a passive process involving the diffusion of small molecules (< 900 daltons) from cell to cell. A number of methods are available whereby this process may be detected and, in most cases, accurately quantified. These methods may be placed into two categories; invasive and non-invasive.

Unique to the invasive methods is the technique of electrical coupling whereby microelectrodes are inserted into cells linked either directly, or indirectly via a

chain of cells. Electric (i.e., ionic) current pulses are then passed between the interior of one cell and the external medium, and the resulting voltage drops are measured across the membrane of that cell and of a neighbouring cell. In cells coupled by junctional membrane channels, a substantial fraction of the current passes into the neighbouring cell (Socolar and Loewenstein, 1979). This technique provides an extremely sensitive test for junctional communication, but unfortunately saturates at quite low levels of communication and therefore only conveys quantitative information when the degree of junctional communication within a system is low.

It is possible to obtain quantitative information about junctional coupling by measurement of electrical conductance. However, this is a more difficult technique to apply than the measurement of junctional coupling since additional factors such as non-junctional membrane resistance have to be taken into account, and in practice such measurements have only been possible with very simple systems such as cell pairs or short cell chains (Loewenstein, 1979; Socolar and Loewenstein, 1979). For example, by applying electrical pulses to embryonic Xenopus muscle cells micromanipulated into contact, it has been demonstrated that the amplitude of the current passing into the recipient cell increases in a stepwise manner with time, indicating that junctional channel formation in this system is an additive process rather

than an all-or-nothing event (Chow and Young, 1987).

The most widely used tool for detecting variations in junctional communication competence is the intracellular transfer of fluorescent tracers. This method gives direct and quantitative information about junctional permeability for various permeant molecular species. The usual means of introducing a fluorescent tracer to a cell is by microinjection, and the dye employed for such studies was originally fluorescein since it is strongly fluorescent and inexpensive (Loewenstein, 1966). However, fluorescein is not an ideal tracer as it is able to traverse the cell membrane with ease and thus diffuses rapidly into the extracellular medium and into neighbouring cells. For this reason it has now been largely superseded as a junctional tracer by the 6-carboxy-form which is more hydrophilic than fluorescein and is therefore retained longer by cells (Loewenstein, 1979). The great advantage of this method over electrical coupling lies in the fact that it can be applied to complex systems such as entire embryos, and the resolution is such that the progress of the dye can be monitored in space and in time using a darkfield microscope linked to an image intensifier system and video camera, allowing the rate of junction-mediated transfer within a given system to be computed (Pollack, 1976; Beigon *et al.*, 1987). An obvious limitation is that the technique is only applicable to transparent tissues, unless it is not necessary to maintain the integrity of

the tissue under investigation, in which case the passage of dye can be determined by taking sections for examination (Pitts *et al.*, 1986). Using fluorescent dye transfer, it has proved possible to delineate communication compartments within structures such as developing embryos and insect imaginal discs (see section 1.20). The technique of tagging synthetic peptides with fluorescent tracers, so creating fluorescent probes spanning a wide range of molecular sizes, has enabled workers to accurately determine the normal size limit of junctional permeation (Simpson *et al.*, 1977; Loewenstein, 1979).

A method which does not require microinjection, and which is applicable to whole organs or tumours, is the introduction of tracer molecules via the cut end of a tissue (Imanaga, 1974). The cells are loaded with a fluorescent or radioactive tracer through the cut end in Ca-free medium and the tracer may then enter intact cells via junctional membrane. Replacing the medium with Ca^{2+}-containing medium traps the tracer, presumably by closing cell junctions at the cut end. Tracer diffusion throughout the tissue may then be followed. The major problem with this method, as with all other invasive methods, is that the validity of this procedure is dependant upon the assumption that the permeability of the cell junctions at the cut end has not been significantly perturbed by the experimental procedure.

To overcome this problem, and to facilitate measurements of junctional coupling within large populations of cells, a number of non-invasive methods for assessing the permeability of gap junctions have been developed. Rottman and Papermaster (1966) discovered that nonpolar fluorescein esters, such as fluorescein diacetate, dipropionate or dibutyrate, go through the non-junctional cell membrane and, once inside the cells, are rapidly hydrolysed by esterases, yielding free fluorescein. Since the outflow of fluorescein is slower than the inflow of the ester, this method is suitable for loading cells with fluorescein. The problem with this approach is that cells necessarily have to be loaded with tracer and then cocultured with recipient cells, and the time required for junctional contact to be established may be such that fluorescein loss renders the results ambiguous (Sellin *et al.*, 1971; Sellin *et al.*, 1974; Loewenstein, 1979). This problem has been overcome by Goodall and Johnson (1982), who incubated cells in the non-fluorescent, non-polar reagent 6-carboxyfluorescein diacetate (CFDA), which enters the cell and is enzymically converted to the hydrophilic fluorophore 6-carboxyfluorescein. This does not leak significantly from non-junctional membrane.

A simple and accurate method for determining junctional permeability is based upon the phenomenon of metabolic cooperation (section 1.11). The original form of this method involved the coculture of $HPRT^-$ and $HPRT^+$ cell lines incubated in the presence of ^{3}H-hypoxanthine

(figure 1.2). As a consequence of the genetic lesion, HPRT$^-$ cells cannot by themselves convert hypoxanthine to inosine mono-phosphate (IMP) for incorporation into their nucleic acid, but when in junctional contact with wild-type cells, IMP is transferred from cell to cell and is incorporated into the nucleic acid of the HPRT$^-$ cells (see section 1.18). This process is easily visualised autoradiographically, and the competence of the test cell for transferring the metabolite is assessed by counting the number of silver grains overlying recipient cells (Subak-Sharpe *et al.*, 1966). A similar autoradiographic method that does not require mutants has been developed by Pitts and Simms (1977). In this method, donor cells are first labelled with ^{3}H-uridine, washed, and then seeded onto unlabelled cells. After a suitable period of time, the cells are fixed and treated for autoradiography, and grains counted as above. This technique can be applied to many types of cell in culture, and with several precursors apart from uridine, such as fucose, choline phosphate and proline (Pitts, 1977).

A variant of this method, introduced by Fujimoto *et al.* (1971) uses toxic purine or pyrimidine analogues which the wild-type cell, but not the mutant cell, can convert into nucleotides. Thus mutant cells survive in the presence of the toxin while wild-type cells are killed. This phenomenon is known as the "kiss-of-death" (see figure 1.2 and section 1.18). Thus Corsaro and Migeon

FIGURE 1.2

a) Postulated mechanism of metabolic cooperation for nucleotides derived from ^{3}H-hypoxanthine.

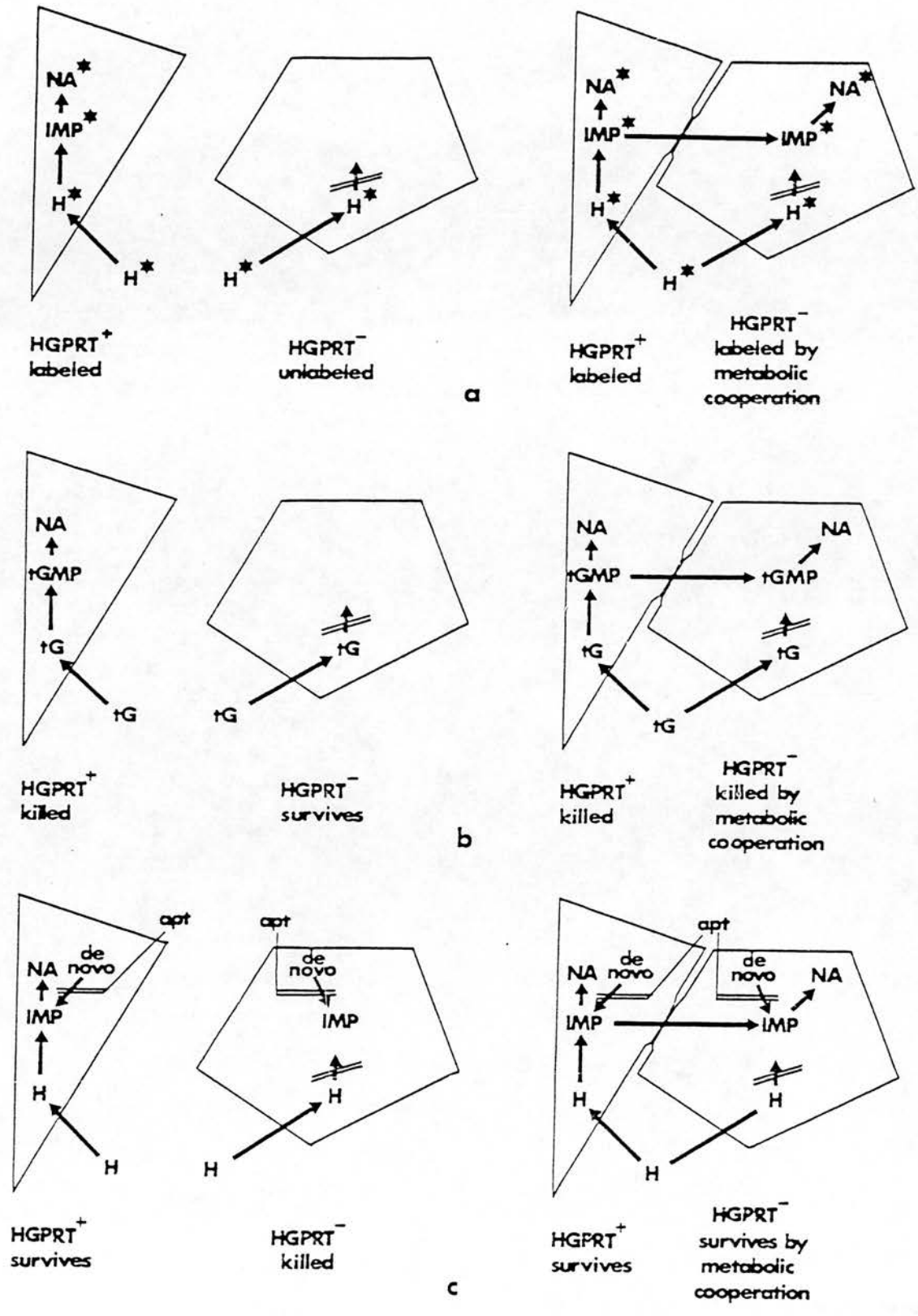
b) "Kiss-of-death" between HPRT^{+} and HPRT^{-} cells in 6-thioguanine.

c) "kiss-of-life" between HPRT^{+} and HPRT^{-} cells in HAT medium.

H = hypoxanthine
apt = aminopterin
tG = 6-thioguanine
NA = nucleic acid

Asterisks denote ^{3}H labelled compounds.

Adapted from Hooper and Subak-Sharpe (1981).


NA
IMP
H
HGPRT+
labeled
HGPRT−
unlabeled
a
HGPRT+
labeled
HGPRT−
labeled by
metabolic
cooperation
tGMP
tG
HGPRT+
killed
HGPRT−
survives
b
HGPRT+
killed
HGPRT−
killed by
metabolic
cooperation
apt
de
novo
HGPRT+
survives
HGPRT−
killed
c
HGPRT+
survives
HGPRT−
survives by
metabolic
cooperation

(1975) used the kiss-of-death assay to determine the communication competence of malignant cells by coculturing wild-type and HPRT$^-$ cells in the presence of 6-thioguanine and measuring the density of donors required to reduce the colony-forming efficiency of the mutants by 50% (the mean lethal density or MLD). Slack *et al.* (1978) used a similar method, the difference being that they quantified their results as a ratio of colony-forming efficiencies in the presence of a standard density of donors.

A related phenomenon is the "kiss-of-life", in which the intercellular transfer of molecules can rescue cells from environments which are otherwise toxic. There have been several demonstrations of this effect. For example, wild-type cells can rescue HPRT$^-$ cells from the toxic effects of a metabolic inhibitor such as azaserine (Fujimoto *et al.*, 1971) or aminopterin (Slack *et al.*, 1978; Hooper and Morgan, 1979b) (see figure 1.2 and section 1.18). Finbow and Pitts (1981) have demonstrated the rescue of proline auxotroph cells by wild-type cells in proline-deficient media. Also Corsaro and Migeon (1976) have shown that wild-type cells in coculture with ouabain resistant cells can be rescued from the toxic effects of ouabain, which inhibits the plasma membrane Na^+, K^+ ATPase responsible for pumping Na^+ ions out of the cell in exchange for K^+ ions, by virtue of junction-mediated exchange of these ionic species between the sensitive cell type and the resistant cell type

(figure 1.3). This rescue was not seen in coculture with L-cells (which had been shown to be defective in metabolic cooperation, section 1.18), or when the sensitive and resistant cells were not in contact with one another.

Various means have been employed to quantify "kiss-of-life" experiments. Pitts (1971) used a mutual rescue technique in which cell growth was only observed if both rescue processes were efficient, so the extent of metabolic cooperation could be determined by counting the number of cells in the coculture. However, where only one cell type is subject to rescue it is necessary to devise some means of distinguishing rescued cells from the large background of rescuing cells. Corsaro and Migeon (1977) dealt with this problem by coculturing cells of different species and estimating the numbers of rescued cells by karyotyping the mixed cultures. A simpler alternative to this makes use of the fact that cells that have been treated with mitomycin C cease to divide while remaining cooperation competent. Thus, if the test cells are seeded onto a layer of mitomycin C-treated rescuing cells, the rescued cells form colonies which can easily be counted (Slack *et al.*, 1978; Hooper and Morgan, 1979b). As a variant of this technique, Nicolas *et al.* (1978) prelabeled HPRT$^-$ cells with ^{3}H-thymidine prior to coculture with wild-type cells in HAT, and used the release of radioactivity into the medium as a measure of cell death. Pitts (1978) devised an elegant means of

FIGURE 1.3

Postulated mechanism of rescue from ouabain toxicity of a sensitive cell by coculture with a resistant cell.

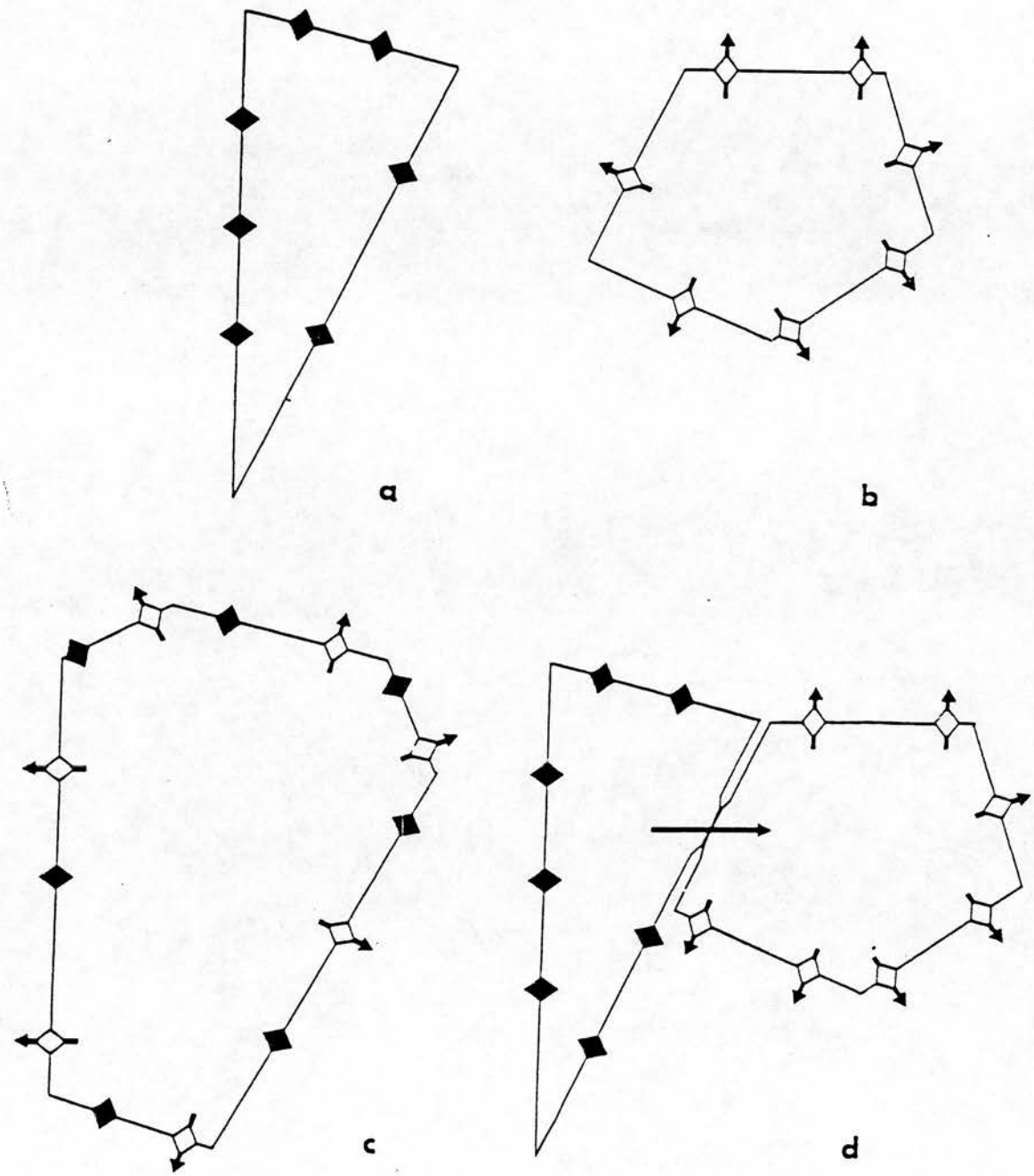
a) Ouabain-sensitive (oua^s) cell. In the presence of ouabain the Na^+, K^+-ATPase (◆) is inhibited and the cell dies due to an inability to pump Na^+ out of the cell.

b) Ouabain-resistant (oua^r) cell. In ouabain, the Na^+, K^+-ATPase of this cell (◇) remains active. Arrows indicate movement of Na^+, which is accompanied by an equal and opposite flow of K^+.

c) Fusion hybrid between oua^s and oua^r cell. The membrane contains the ATPase of both parents so that in ouabain only a fraction of the ATPase is active, resulting in an intermediate level of resistance.

d) oua^r and oua^s cells connected by a junction permeable to Na^+. Since Na^+ can pass freely from one cell to another, the situation is analogous to (c).

Adapted from Hooper and Subak-Sharpe (1981).


a
b
c
d

measuring metabolic cooperation whereby TK^-, ouabain-resistant cells are cocultured with TK^+, ouabain-sensitive cells in the presence of ouabain and ^{3}H-thymidine. ^{3}H-thymidine can only be incorporated if the TK^+ cells are rescued from ouabain toxicity (in which case some ^{3}H-thymidine will also cross as nucleotide to the TK^- cell type and be incorporated there). Thus, the level of metabolic cooperation can be measured by scintillation counting.

Although metabolic cooperation assays are generally easy to quantify and straightforward to perform, the method does have some shortcomings. One difficulty is that of finding adequate controls to show that toxic metabolites (in the case of the "kiss-of-death") or "rescuing" metabolites (in the case of the "kiss-of-life") are not being transported via the extracellular medium. This is normally achieved by the use of an L cell control or by cultivating cells together in the same medium but not allowing them to make contact, e.g. by floating a coverslip of the recipient cell type in the medium above a monolayer of sensitive cells. More seriously, while the identity of the precursor may be known, the identity of the molecule that is actually transferred through the junction can only be inferred since metabolic cooperation is a complex process involving a series of steps of which junctional transfer is only one. It must be borne in mind that metabolic cooperation assays detect the long-term consequences of transfer rather than the transfer itself,

and so the final results could be modified by factors such as differences in the normal nucleotide pool sizes of different cell types. Therefore, although these methods are widely employed, the results must be interpreted with care.

More recently, Vitkausas and Canellakis (1987) have described a novel method for the quantitation of metabolic cooperation. The method depends upon measuring the increase in HPRT activity that occurs within $HPRT^+$ cells cocultured in HAT medium with $HPRT^-$ cells. This is achieved by measuring the uptake of ^3H-hypoxanthine in a scintillation counter. The authors claim that the rise in HPRT activity is a result of the level of the enzyme phosphoribosyl pyrophosphate synthetase (PRPP), which is considerably elevated in $HPRT^-$ cells, being synthesised at a higher level in $HPRT^+$ cells when they are in junctional contact with $HPRT^-$ cells. This could lead to problems with some systems because, as the authors point out, some compounds tend to alter the PRPP pools of the $HPRT^+$ cells disproportionately from the PRPP pools of the $HPRT^-$ cells and so if this method is used to determine the effect of a drug it is advisable to measure the PRPP activity of the $HPRT^+$ cells. Therefore, considering the simplicity and variety of alternative methods, this would seem to be an unnecessarily complex assay, and probably of little practical value.

1.13 GAP JUNCTIONS

Gap junctions are sieve-like structures which permit free diffusion of low molecular weight, water-soluble components between the cytoplasms of coupled cells (for a recent review see Pitts and Finbow, 1986). They are ubiquitous structures, to be found in all cells of metazoan animals with the exception of a few special cases where they are lost in the terminal stages of differentiation. In thin section electron micrographs they appear between cells as regions of closely apposed membrane, separated by a gap 2-4nm in width , hence the term gap junction. In freeze-fracture electron micrographs they typically appear as large plaques composed of numerous individual junction particles (connexons). These particles may be arranged in a random manner or as a quasi-crystalline structure with hexagonal symmetry, and there are numerous publications claiming a correlation between the geometry of particles within a plaque and the functional state of the junctions, i.e. whether they are open or closed, at the time of preparation (Hirokawa and Heuser, 1982; Page and Upshaw-Early, 1981; Page *et al.*, 1983; Perrachia, 1980). The current concensus of opinion is that the crystalline arrangement represents closed junctions, but that the change to crystalline form may be a long-term consequence of uncoupling rather than the uncoupling event itself (Hanna *et al.*, 1986). Some earlier results indicating that the crystalline form represented the open state may

be explained by the observation of Raviola *et al.* (1980) that different preparation techniques may themselves give rise to alterations in particle spacing and so at least some of the published results may be artefactual. Freeze-fracture electron microscopy has also provided valuable information concerning the subunit structure of the connexon (Peracchia, 1980; Zampighi and Simon, 1985; Hanna *et al.*, 1985, and see below).

Structural analysis of gap junctions has been greatly facilitated by the fact that gap junctions are relatively easy to isolate. Gap junctional plaques in isolated fragments of plasma membrane display a characteristic hexagonally-arranged crystalline arrangment similar to those observed *in vivo*, except that in isolation they are more closely packed (Makowski *et al.*, 1977; Caspar *et al.*, 1977). It has been possible therefore for a number of workers to apply X-ray and electron diffraction techniques to such preparations (Makowski *et al.*, 1977; Caspar *et al.*, 1977; Unwin, 1986), and these studies show that the individual connexon is made up of six similar, probably identical, subunits, arranged axially around a 2nm water-filled channel. These subunits are rod-shaped and appear to be of the order of M_r20,000 to 30,000 (20 to 30K) in size. Although the resolution of these techniques is not high enough to identify any secondary structure, it is known that each subunit is tilted at a slight angle to the long axis of the connexon, and that the angle of tilt can be decreased (in isolated

junctions) by uncoupling procedures such as loading with Ca^{2+}, causing the cytoplasmic ends of the connexon to pinch together. It has been suggested that this conformational change may form the basis of a channel gating mechanism (Zampighi and Unwin, 1979; Unwin and Zampighi, 1980; Unwin and Ennis, 1984).

Gap junctions are unusually resistant to proteases such as collagenase and trypsin, detergents such as Triton X-100, and to chaotropic agents like urea (Goodenough, 1976; Finbow *et al.*, 1983). These properties have been variously exploited to facilitate the isolation and purification of the junctional protein. The original isolation procedure involved isolation of plasma membranes followed by extraction with detergents (Benedetti and Emmelot, 1968; Goodenough and Stoekenius, 1972). The resulting isolate could then be pelleted and the junction-related proteins separated from other detergent resistant components by sucrose density gradient ultracentrifugation. Various modifications to this procedure have been introduced to remove contaminants, such as trypsin or urea treatment of plasma membranes prior to density gradient centrifugation (Goodenough, 1976; Culvenor and Evans, 1977). In the absence of any functional assay for the presence of junctional proteins, preparations are then examined under the electron microscope to assess purity. Although it is easy to spot typical junctional structures by this method, it is purely qualitative and does not rule out

the presence of contaminants, and for some time there were a number of different potential candidates for junctional protein with no real consensus as to which one represented the actual protein and which ones were contaminants (Robertson, 1981). This was the situation up until 1979, when three different groups reported the identification of a 27K protein as a major component in junctional preparations (Hertzberg and Gilula, 1979; Henderson *et al.*, 1979; Finbow *et al.*, 1980). Being similar in size to the subunit visualised by electron and X-ray diffraction, and to the lens major intrinsic protein (26K, MIP26) which was consistently found in junctional preparations from lens tissue, this would appear to be the most likely candidate for the junctional protein, and consequently considerable attention has been focused upon this protein. In 1983, however, Finbow *et al* announced the discovery of a protein with a molecular weight of 16K in junctional preparations isolated by a more gentle technique than had previously been applied (Finbow *et al*, 1983), and it has since emerged that this band of protein was not discovered in previous preparations because the heat solubilisation procedures used prior to loading samples onto SDS-PAGE gels caused it to form multimeric structures which were not able to enter the gel and therefore were not observed (Finbow *et al.*, 1987). Isolates of this protein as visualised by negative staining under the electron microscope have the appearance of the classic gap junction structure (Finbow *et al.*, 1987), and the authors suggest that the

discrepancy between the size of this protein on SDS-PAGE gels and that of the connexon subunits as visualised by other techniques may be due to the connexon subunit being a dimeric form of the 16K protein. Alternatively, they suggest that the discrepancy may be explained by the fact that very hydrophobic proteins have a tendency to migrate anomalously on SDS-PAGE gels, and may therefore give rise to misleading results. Initial suggestions that the 16K protein was a fragment of the 27K protein have been dispelled by peptide mapping analysis which has shown that the two proteins are unrelated. However, Zervos *et al.* (1985) claim that the major component of the gap junction is a 27K protein which can dimerise to 47K, and that tryptic peptide mapping of radio-iodinated proteins reveals a 65K component which is similar to the 27K and 47K proteins but which appears to yield, on tryptic digestion, several different peptides, suggesting to them that the 65K protein could be a precursor molecule. Also, work by Ohla *et al.* (unpublished observations cited in Finbow *et al.*, 1987) indicates that while the tryptic peptide maps of the 16K and 27K proteins are unrelated, a tryptic peptide map of the 65K protein shows peptides that are also present in both the 27K and 16K preparations. They conclude that there may be a parent gap junction protein that gives rise to the 27K and 16K polypeptides and suggest that the 16K protein may derive from a regulatory subunit of the gap junction protein. Whether their 16K protein is identical to the one isolated by Finbow *et al.* remains to be established.

Thus for the present, the only definite conclusion which can be drawn from the above investigations is that there is a 27K and a 16K protein component associated with gap junctions. Which one represents the actual structural unit, or whether they interact in some manner, is not clear.

It has also been found that the lens junctional protein, MIP26, is unrelated to the 27K protein (Hertzberg *et al*, 1982) and it is now thought that the primary function of MIP26 is to hold the individual lens cells tightly together in order to maintain the optical properties of the lens. Since lens cells are clearly coupled it may be that this protein also performs an intercellular communication function, although it is possible that there are other junctional proteins dispersed among the particles of MIP26.

1.14 EVOLUTIONARY CONSERVATION OF THE GAP JUNCTION PROTEIN

The gap junction isolation method devised by Finbow *et al* (1983) was originally conceived because of a need to isolate gap junctions from small numbers of cells in culture. This has proved to be of great utility since it has now become possible using this methodology to isolate junctional proteins from a diverse array of sources previously uninvestigated due either to a low yield of

junctions or simply to difficulty in obtaining sufficient material for analysis. Having overcome this problem to some extent, Dr Finbow and his colleagues have isolated gap junctions from a number of vertebrate and invertebrate sources and have compared these proteins immunologically and by 2-dimensional peptide mapping (Finbow *et al*, 1983 and 1984). These studies have revealed that the 16K protein is present in all vertebrate sources tested (mouse liver, kidney, brain, heart, lung and uterus; from the liver of other mammalian, avian and amphibian species; and from a wide variety of tissue culture cells), and that immunologically related proteins are present in isolates from the Norway lobster (18K protein) and the Octopus (19K protein).

1.15 CHEMICAL INHIBITORS OF METABOLIC COOPERATION

The functions of proteins *in vivo* are frequently investigated by observation of the effects of applying specific inhibitors to the system. However, despite the fact that a number of treatments have been shown to inhibit junctional communication, this approach has been severely hampered to date by the fact that no agent is yet available which specifically interacts with the gap junction alone (with the possible exception of some antibody preparations). Inhibitors have therefore proved to be of greater utility for the investigation of, for

example, structural changes caused by uncoupling, than for determination of the effects of uncoupling upon cellular functions such as differentiation.

Inhibition of junctional communication was first observed under conditions of elevated intracellular Ca^{2+} concentration (Loewenstein 1966, 1981). More recently Turin and Warner (1977) and Spray *et al* (1981; 1982) have proposed that uncoupling may be a consequence of increased internal H^{+} concentrations. However, it is difficult to separate the effects of high internal calcium from the effects of cytoplasmic pH as these processes are highly interdependent. These phenomena, particularly Ca^{2+} loading, have been exploited for investigations of the structural consequences of uncoupling (Perrachia, 1980). High intercellular calcium levels cause the junctional particles to aggregate tightly into crystalline arrays similar to those seen after other uncoupling treatments (Peracchia and Peracchia, 1980). Perrachia and Girsch (1985) have suggested that the effect of calcium upon junctional communication may be mediated by calmodulin binding to a specific site on the carboxyterminal region of the connexon. It has also been shown that very low extracellular levels of calcium cause cells to decouple (Loewenstein, 1966), although this is a non-specific effect arising as a result of alterations in cell adhesion properties.

Junctional communication is inhibited by high levels (10^{-4}M) of retinoic acid (Pitts *et al* 1981, 1986; Hamilton and Pitts 1981). This inhibition is not accompanied by any alteration in junction structure (as assessed by freeze-fracture), or by a reduction in the quantity of junctional protein isolable from treated cells. In fact, Pitts *et al* (1986) have shown that junctional area and junctional protein yield actually increase. Also, the inhibition of cooperation is rapidly established and rapidly reversible. These observations show that retinoic acid acts by closing the junctional channels rather than preventing formation or causing breakdown. The precise mechanism of retinoic acid-mediated inhibition is not understood, although it has been suggested that it may be similar to the mode of action of the alcohols 1-Heptanol and 1-Octanol (Pitts *et al*., 1986).

The aliphatic alcohols 1-heptanol and 1-octanol are powerful inhibitors of junctional communication (Johnston *et al*., 1980; Bernardini *et al*., 1982, 1984). In all systems tested heptanol and octanol uncouple junctions within 15 minutes and recoupling occurs approximately 15 minutes after washout. In a freeze-fracture study of rat stomach and pancreas gap junctions Bernardini *et al* (1984) have determined that particle density reversibly decreases after 10-15 minutes exposure to heptanol-Tyrode's solution in the case of stomach, and after 30 minutes exposure for pancreas. This is in good

agreement with the time-course of heptanol-induced inhibition of electrical coupling in these tissues and it is tentatively suggested that heptanol could act by direct interference with the particle organization of gap junctions. Although the precise mechanism of heptanol and octanol inhibition has yet to be elucidated, it has been determined that the site of action is likely to be upon the extracellular face as internal perfusion of the crayfish septate axon with heptanol or octanol has no effect upon junctional communication (Johnston *et al.*, 1980; Ramon *et al.*, 1985). Also it has been determined that the uncoupling action of heptanol and octanol is unlikely to be due to the alcohols dissolving into the membrane lipids and influencing their fluidity as ethanol, 1-butanol, 1-hexanol, 1-nonanol and 1-decanol have no effect upon junctional permeability (Ramon *et al.*, 1985). Wojtczak (1985) has observed that octanoic acid also produces uncoupling but unlike octanol the effect is only slowly reversed upon washout. This leads to the conclusion that the presence of the hydrophilic group at the end of the hydrophobic side-chain is important, and he suggests that the uncoupling effect produced by heptanol and octanol may be due to the participation of the hydroxl group in weak, reversible hydrogen bonds. If this is the case then the poor reversibility of octanoic acid may be explained by the formation of a more stable ionic bond by virtue of the terminal carboxyl group.

The tumour promoter TPA (4β-phorbol 12-myristate 13-acetate) inhibits metabolic cooperation by reducing the number of junctional particles over a period of time (Yancey *et al.*, 1982; Walder and Lutzelschwab, 1984; Pitts and Burk, 1987). Pitts and Burk found that inhibition of metabolic cooperation by TPA was most marked in cell lines whose natural rate of junction formation was low. Also TPA is effective in reducing coupling at a wide range of concentrations (10^{-9} to 10^{-6}M), suggesting to the authors that it may act by inhibiting or reducing the level of some intermediate activity which in turn has a partial effect upon junctional coupling. They postulate that TPA causes a reduction in the rate of junction formation, and that cells with an inherently high rate of turnover of junctional particles are able to produce sufficiently large quantities of gap junctions to maintain a nearly normal level of intercellular communication.

A number of other substances have been claimed to exert some inhibitory effect, directly or indirectly, upon junctional coupling. For example, the tumour promoters aplysiatoxin and debromoaplysiatoxin (Jone *et al.*, 1987); dopamine (Neyton *et al.*, 1985); halothane (Wojtczak, 1984); and transformation with Rous Sarcoma virus (Atkinson *et al.*, 1986).

As previously intimated, interpretation of the results of treating cultures with inhibitory agents is complicated

by our poor understanding of the mechanisms involved, and by the possibility of unrelated secondary effects. A useful approach to elucidating some of these mechanisms might be to select variant cell lines resistant to the inhibitory effects of various agents. The viability of this proposition has been demonstrated by Smith *et al* (1986) who selected an EC cell line (PTmr0, derived from PSA4TG12) resistant to the inhibition of metabolic cooperation by 10^{-4}M retinoic acid. Selection of a battery of such lines against various inhibitors would allow direct comparisons to be made between the mode of action of different inhibitors. For example, does retinoic acid inhibit junctional coupling by the same route as octanol and heptanol? A preliminary report on the effect of heptanol upon junctional communication in PTmr0 is presented in chapter 5 of this thesis. Variants of this nature selected from totipotent ES cells might also answer questions about the physiological significance of junctional gating.

1.16 ANTIBODIES RAISED AGAINST GAP JUNCTIONS

A more promising approach to the investigation of gap junction function by inhibition of communication is the production of gap junction-specific antibodies. The original study of this type was carried out by Warner *et al* (1984), who raised two rabbit polyclonal antibodies (antibodies A and B) to the 27K rat liver junctional

protein, and were able to demonstrate that the injection of either of these antibodies into the Xenopus embryo resulted in inhibition of metabolic cooperation. A control antibody, antibody C, raised against an extracellular matrix protein secreted by the rat liver and by Xenopus embryos, caused no perturbation in metabolic cooperation. Also, metabolic cooperation was unaffected by IgG extracted from the rabbit serum prior to immunisation. The effect of these antibodies was to perturb development of the Xenopus embryo (see section 1.20). Critics of this set of experiments have questioned the specificity of these antibodies, as not only do they react with the 27K protein, but also there is cross-reaction with a 47K and a 54K species. The 47K protein is claimed to be a dimeric form of the 27K protein but the origin of the 54K protein is less clear. However, Fraser *et al* (1987) claim that the 54K protein is another dimeric form of the 27K protein produced under certain purification conditions. These antibody preparations have since been used to investigate the role of gap junction-mediated communication in the mechanism of head inhibition in Hydra (Bode *et al*., 1987; Fraser *et al*., 1987) (see section 1.20).

1.17 STIMULATION OF METABOLIC COOPERATION

Treatment of cells with cAMP or its derivative dibutyryl cAMP, or elevation of endogenous levels of cAMP by serum

deprivation or lowering of cell density in 3T3 cells, has been shown to produce an increase in junctional permeability (Flagg-Newton, 1980; Flagg-Newton *et al.*, 1981). Hormone treatments which raise the endogenous level of cAMP have also been shown to elicit a simultaneous increase in junctional coupling (Radu *et al.*, 1982). Coupling is increased in Drosophila salivary glands exposed to the moulting hormone β-ecdysone, which raises cAMP levels (Hax *et al.*, 1974). An instance in which a hormone produces an elevation in coupling without a change in cAMP levels is found in cultured superior cervical ganglion neurons (Kessler *et al.*, 1984). When these cells are dissociated and cultured in the presence of serum, electrotonic coupling is rare. However, culture in serum-free defined medium supplemented with insulin, or when insulin is added to serum-containing medium, results in an increase in coupling. This is a long-term effect which develops over a number of days and has been shown to be independent of cAMP.

In various cell lines, db-cAMP or phosphodiesterase inhibitors increase electrical and dye coupling and also increase the incidence of gap junctions (Flagg-Newton *et al.*, 1981; Azarnia *et al.*, 1981). In superior cervical ganglion, db-cAMP exposure leads to rapid development of electrotonic coupling which peaks within 12 hours (Spray and Bennett, 1985). The phosphodiesterase inhibitor theophylline, which raises intercellular levels of cAMP, also stimulates junctional communication. Smith (1984)

found that treatment of cooperation-defective cell lines derived from the EC line PSA4TG12 with either db-cAMP or with theophylline restored levels of intercellular nucleotide transfer to those of the parental control, with the exception of one particularly cooperation-deficient variant, PT2md1, whose level of nucleotide transfer was raised to an intermediate value.

Weiner and Loewenstein (1983) investigated the metabolic cooperation phenotype of a mutant Chinese Hamster Ovary (CHO) cell line completely deficient in the production of a cAMP dependant protein kinase phosphorylating protein kinase I (I^-) and with reduced activity of a related protein, phosphorylating protein kinase II. They found that metabolic cooperation was significantly reduced in this cell line as compared to the parent line. Furthermore, spontaneous revertants to the mec^+ phenotype were also restored in their ability to produce phosphorylating protein kinase I. A phosphorylating protein kinase II defective mutant line showed no reduction in metabolic cooperation. Prompted by these findings, they then loaded I^- cells with a purified preparation of the catalytic subunit of phosphorylating protein kinase I, with the result that metabolic cooperation was fully restored to the parental level. They also report that I^- mutants have fewer gap-junctional membrane particles than the wild-type parent. These observations have led the authors to suggest that a step of protein phosphorylation may directly intervene in

the assemblage of the junctional channel.

1.18 SELECTION OF METABOLIC COOPERATION-DEFECTIVE CELL LINES

The ability of cells to exchange nucleotides and other small molecules via gap junctions has led to the development of a number of selective techniques designed to isolate variant cell lines with altered junctional communication abilities. The first demonstration of this was made by Wright *et al* (1976), who selected a cooperation defective (mec^-) cell line from a polyoma transformed Syrian hamster cell line defective in TK (thymidine kinase), dCK (deoxycytidine kinase) and HPRT (hypoxanthine guanine phosphoribosyltransferase). By virtue of the TK deficiency this line was resistant to growth in the presence of BUdR (bromodeoxyuridine) followed by exposure to blue light. When co-cultured with TK^+ cells, they became sensitive through the "kiss-of-death" mechanism (figure 1.2). This phenomenon was utilised as a selective technique to enrich cultures for poorly cooperating variants. After 46 rounds of selection, a variant was recovered (mec^-1A) which was not only deficient in its ability to transfer radiolabelled nucleotides (figure 1.2) from cell to cell but also was different from the parent line in size, morphology, growth rate and karyotype. It was shown that the reduced level of nucleotide transfer was attributable to a

junctional transfer defect, and not to an alteration in nucleotide metabolism. This transfer defect proved to be reversible by treatment with dibutyryl cAMP and theophylline. Polypeptide mapping revealed at least 11 differences between the protein profiles of the parental line and mec-1A, 6 of which reverted to the wild-type phenotype upon treatment with dibutyryl cAMP and theophylline, making them obvious candidates for involvement in the cooperation defect.

Clearly, such a lengthy selection procedure is undesirable as not only is it time-consuming to perform, but also and more importantly it allows a build-up of secondary defects, making results difficult to interpret. The efficiency of killing was substantially increased by Slack *et al* (1978), who derived the mec$^-$cell line R5/3 from the HPRT$^-$ EC cell line PC13TG8. This is a feeder-independent line which is capable of differentiation in tumours *in vivo*, and of differentiation in monolayer culture in response to retinoic acid. The method employed for this selection exploited the fact that the HPRT deficiency confers resistance to the purine analog 6-thioguanine (tG), as in the absence of HPRT the cell is unable to convert tG to tGMP, thus preventing its incorporation into the nucleic acid. When co-cultured with HPRT$^+$ cells in the presence of tG, the tGMP produced by the wild-type cells is able to pass via gap junctions into the variant cells, where it is incorporated into the nucleic acid with lethal

results (figure 1.2). Obviously, an HPRT$^-$cell defective in gap junction-mediated intercellular communication would be expected to survive this treatment. The isolation of R5/3 was thus achieved in 5 rounds of selection by co-culture of PC13TG8 with its parent line PC13 in the presence of 10μg/ml tG. As well as the metabolic cooperation defect, R5/3 differed from PC13TG8 in that it was subtetraploid, exhibited increased resistance to tG and was restricted in its *in vivo* differentiation capacity. The response of R5/3 to retinoic acid, however, remained similar to that of PC13TG8. The mec$^-$phenotype of R5/3, initially determined by ^{3}H-uridine nucleotide transfer, was confirmed by subsequent experiments which showed that it was also defective for the transfer of amino acids and alkali metal ions (figure 1.2) (Hooper and Morgan, 1979a). Also it was shown by Hooper and Parry (1980) that R5/3 has a decreased total gap junction area per unit cell volume and an increased surface area of microvilli per unit cell volume as compared to the parent line. Somatic cell hybridisation of R5/3 cells with mouse L cells gave rise to hybrid lines restored in their ability to cooperate, thus indicating the existence of at least two loci involved in metabolic cooperation (McDonald, 1982).

It must be stressed at this point that the metabolic cooperation deficiency was incomplete in that in nucleotide transfer experiments there is always a small number of heavily labelled R5/3 recipient cells, and

there is some colony survival, albeit at a low level, in ouabain rescue experiments. This may be explained by a probability model of metabolic cooperation (Gaunt and Subak-Sharpe, 1979), which postulates that the phenotype of mec$^-$ cell lines is due to a reduction in the probability of junction formation when cells come into contact rather than an absolute, and fixed, reduction in junctional communication. Thus on occasion a cooperation deficient cell line may participate in junctional communication at a normal level. The term mec$^-$ is therefore somewhat misleading and strictly speaking such cell lines should be referred to as cooperation defective lines.

In order to relate the phenotypic changes in R5/3 to its cooperation deficiency, Hooper and Morgan (1979b) selected a revertant line, H2T12, restored in ability to cooperate. This selection procedure made use of the toxicity of HAT (hypoxanthine, aminopterin and thymidine) medium to HPRT$^-$ cells but not to HPRT$^+$ cells. Aminopterin binds irreversibly to the enzyme folate reductase, blocking both the endogenous purine synthesis pathway and the conversion of deoxyuridylic acid to thymidylic acid in the pyrimidine synthesis pathway. This therefore makes the cell reliant upon the presence of preformed precursor molecules such as hypoxanthine and thymidine for the synthesis of nucleic acid precursors through the alternative purine and pyrimidine salvage pathways using the enzymes HPRT and TK respectively. If

HPRT$^-$ cells are cultured on a mitomycin-killed feeder layer of HPRT$^+$ cells in the presence of HAT, their survival is dependant upon the receipt of nucleic acid precursors, specifically IMP, from the cells of the feeder layer. Since this can only occur via gap junction-mediated intercellular communication it follows that only mec$^+$ cells will survive these conditions. This phenomenon is commonly referred to as the "kiss-of-life" or HAT rescue (figure 1.2).

H2T12 was isolated in this way by one round of HAT rescue selection. In both nucleotide transfer and ouabain rescue assays it was found that metabolic cooperation was restored to normal levels, indicating that they have a common genetic basis. Also, the area of gap junctions and microvilli were restored to their wild-type values, lending further support to the contention that the metabolic cooperation defect is gap junction mediated and suggesting that in the case of R5/3 the lesion was cytoskeletal. The karyotype of H2T12 was similar to that of R5/3, as was the increased resistance to tG and the inability to differentiate *in vivo*, indicating that these were secondary defects unrelated to the metabolic cooperation lesion.

In an attempt to establish a correlation between the differentiation capacity of EC cells and their metabolic cooperation phenotype, a series of cooperation deficient variants have been selected from the pluripotent EC line

PSA4TG12, an HPRT$^-$ derivative of PSA4 (Smith *et al*, 1986). PSA4TG12 is a feeder-dependent cell line which differentiates extensively *in vivo* and *in vitro*. By tG kiss-of-death a series of cooperation defective clones were obtained, all of which were karyotypically similar to PSA4TG12 but restricted in their capacity to form differentiated embryoid bodies. One of the cooperation defective variants, PT2md1, was used as starting material for a "kiss-of-life" selection. This selection was based upon the ouabain rescue assay, whereby ouabain sensitive cells are rescued from ouabain toxicity by culture upon a ouabain-resistant feeder layer (figure 1.3). This resulted in the isolation of a cell line, PT2mo1, which was rescued at high frequency in the ouabain rescue assay but which performed poorly when assayed by uridine nucleotide transfer (Smith T.A., 1984). It was thought at the time that this may have been a mec$^-$ ouabain resistant variant but subsequent work has shown that this is not the case, and that it may in fact cooperate at higher levels with the STO fibroblast line used as the feeder layer for selection than with itself (Hooper M.L., pers. comm.). This is not an unprecedented situation, as the mouse L cell line has also been shown to cooperate at a low level homotypically whilst being able to form heterotypic contacts efficiently with a number of other cell lines (Gaunt and Subak-Sharpe, 1979). A true revertant, PT2mh1, has since been selected from PT2mo1 by HAT rescue (Smith T.A. and Hooper M.L., pers. comm.). This cell line is restored in its ability to transfer

uridine nucleotides homotypically. It also has a karyotype similar to that of PSA4TG12. Its differentiation phenotype with regard to embryoid body formation is, however, identical to that of PT2md1 and so it seems that these lines also have suffered a secondary lesion deleterious to differentiation. A causal link therefore was not established between metabolic cooperation and differentiation capacity, although a statistical analysis of the series of cooperation defective variants indicated that there was a weak association between the reduction in cooperation and loss of differentiation capacity. However, this was inconclusive and was confounded by the much stronger association found between mutagenesis with MNNG and loss of differentiation capacity (Smith *et al*, 1986).

A different approach to investigating the relationship between metabolic cooperation and developmental processes was taken by Janet Smith (1984). She performed somatic cell hybridisations between PSA4 cells and a ouabain resistant derivative of R5/3, R5/3OA. A series of hybrid lines were isolated, all of which were phenotypically similar to PSA4 both with respect to metabolic cooperation and differentiation phenotype. One of these lines, PR3, was chosen for kiss-of-death selection and a thioguanine-resistant derivative, PR3Tg12, was selected to facilitate this. PR3Tg12 was capable of forming cavitated embryoid bodies, but not differentiated tumours. One round of kiss-of-death selection upon

PR3Tg12 produced the cooperation-defective line Kd1a, which was subsequently cloned to yield the line Kd1a.6. Characterisation of Kd1a.6 showed that it was capable of forming cavitated embryoid bodies at a low frequency *in vitro* and, like PR3Tg12, was incapable of *in vivo* differentiation in tumours. Therefore the reduction in metabolic cooperation in Kd1a.6 lines did not prevent embryoid body differentiation. It should be noted, however, that a role for metabolic cooperation in the differentiation process is not excluded by this set of experiments as Kd1a.6, like cooperation defective lines previously isolated, showed only a reduction in metabolic cooperation and not a complete deficit. Also it may be of significance that the extent of differentiation in embryoid bodies was somewhat reduced as compared to the parental hybrid line. It may be that differentiation is also a probabalistic event, and that one of the parameters determining the likelihood of initiation of a particular differentiation pathway is the extent of metabolic cooperation. This idea is developed further in section 3.8 of this thesis.

1.19 THE ROLE OF INTERCELLULAR COMMUNICATION

The ubiquity of gap junctions throughout the majority of animal tissues (exceptions being skeletal muscle, many nerve cells, and blood cells) argues for their importance to cellular function. However, despite intensive research

into gap junctions and metabolic cooperation, few definite conclusions have been reached as to their role *in vivo*. Indeed, most of the current ideas about the role of gap junctions in normal tissues are predictions or speculations based on observations of junctional permeability made in tissue culture and isolated organs (Pitts, 1980). For example, in excitable tissues they can provide pathways for the intercellular transmission of electrical impulses as evidenced by the synchronisation of contractions of cultured myocardial cells when they come into contact (De Mello, 1977).

In vivo, junctional communication could provide a useful pathway for nutrient transfer in the form of energy-rich intermediates such as sugar phosphates or nucleoside phosphates, or it may allow homeostasis and coordinate control of cellular activities to be governed within communication compartments by concentrations of small cytoplasmic molecules (Sheridan *et al.*, 1979; Petersen, 1985). Also, it might provide communication pathways for the intercellular control of proliferation and differentiation (Sheridan, 1976; Loewenstein, 1979; Pitts and Finbow, 1986).

There has been much speculation about possible roles for gap junction mediated communication in the control of cell proliferation (Loewenstein, 1968; 1979; Sheridan, 1976). Since a fundamental property of tumour cells is unrestrained proliferation, it has been postulated that

alterations of junctional permeability may play a role in tumourigenesis (Loewenstein, 1966; 1968). The state of the junctions in various solid tumours and cultured tumour cells has been studied morphologically and physiologically, and it has been found that in many cases (but by no means all) cellular transformation is associated with a reduction in junctional permeability (Atkinson *et al.*, 1986). Also it has been shown that the growth of various chemically and virally transformed cell types in culture is inhibited when they are in direct contact with normal cell types (Mehta *et al.*, 1986), suggesting that some form of regulatory message may pass from normal to abnormal cells in this example. The tumour promoter TPA is known to inhibit junctional communication, and the possibility that the tumorigenicity of TPA may be a consequence of inhibition of junctional communication is an attractive one, supported by the observation that metabolic cooperation is frequently reduced in tumours (Loewenstein and Kanno, 1967) and in transformed cells (Borek *et al.*, 1969) as compared to normal cells. It has been suggested that the reduction in communication with surrounding cells could be a required step in the release of the initiated cell from normal growth control (Yotti *et al.*, 1979). This would seem at first sight to be at odds with the observation that retinoic acid, which can also inhibit junctional communication (Pitts *et al.*, 1986), antagonises TPA-mediated tumour promotion (Bollag, 1972). However, the inhibition of metabolic cooperation by

retinoic acid is manifest at grossly unphysiological, and cytotoxic, levels (see section 1.15) and given the wide-ranging effects of retinoic acid upon cellular functions it is clearly possible that this suppression of tumour promotion may be mediated by an unrelated mechanism. Nevertheless this does not rule out a role for retinoic acid in junction-mediated modulation of cellular function *in vivo*, as it may be that certain cell types are unusually sensitive to junctional uncoupling by retinoic acid, or alternatively that lower concentrations of retinoic acid can reduce metabolic cooperation sufficiently to elicit a response in some systems. Therefore, although the precise relationship between junctional communication and tumourigenesis has yet to be elucidated, the evidence for a link between the two, at least in some instances, is persuasive.

It has also been proposed that in some systems a step in tumorigenesis might be the establishment of junctional contact between cell types which are normally not coupled. Primary cultures of normal breast fibroblasts and epithelial cells do not communicate with each other in mixed cultures although they do form homotypic contacts (Fentiman *et al.*, 1976). In the normal adult breast the two cell types are separated by a basement membrane but invasion of this membrane, characteristic of malignant cells, allows contacts to form between the two and it is speculated that this breakdown of the communication barrier might be a stage in tumourigenesis

(Fentiman *et al.*, 1976).

1.20 JUNCTIONAL COMMUNICATION IN DEVELOPMENT

The transmission of small molecules across fields of cells has obvious attractions as a mechanism for the regulation of developmental processes. Among the first to speculate that gap junction mediated communication might be an important requirement in differentiation and morphogenesis were Furshpan and Potter (1968). Since then, this proposal has been reiterated by many investigators (reviewed in Hertzberg *et al.*, 1981), and although the intercellular signalling mechanisms that control development are still largely unknown, there are good reasons for considering the suggestion carefully.

The principal evidence that gap junctions are important in development is that they appear at embryonic stages when cell-to-cell interactions leading to cellular determination occur (suggesting a role in signal transmission) and that they are absent or less permeable in regions of embryonic tissue destined to form different structures (suggesting that they restrict regulatory signals to the appropriate developmental compartment).

The earliest event in development is the maturation of the oocyte within the Graafian follicle. The developing oocyte is surrounded by follicle cells which form

extensive junctional contacts both with the oocyte (Albertini and Anderson, 1974; Gilula *et al.*, 1978) and with the surrounding cells of the cumulus oophorus, which in turn are junctionally coupled to the peripheral cells of the follicle (the granulosa cells) (Albertini *et al.*, 1975). These junctional contacts appear to be required for the transfer of many nutrients to the oocyte (Colonna and Mangia, 1983; Heller *et al.*, 1981; Moor *et al.*, 1980). Also, autoradiographic studies of the cell to cell transmission of ^{3}H-cAMP (Beers and Olsiewski, 1985), and quantitative freeze-fracture studies (Larsen *et al.*, 1987) have established a correlation between meiotic resumption following human chorionic gonadotrophin injection, and down-regulation of junctional conductance between the membrana granulosa and cumulus oophorus cells, suggesting that regulatory signals responsible for maintaining the oocyte in a condition of meiotic arrest pass via gap junctions into the oocyte, and that meiosis commences upon the cessation of signal transmission. The most likely candidate for such a signalling substance is cAMP (Beers and Olsiewski, 1985). This contention is supported by the observation that cumulus-oocyte complexes removed from their follicles and placed under tissue culture conditions will resume maturation spontaneously in the absence of hormones. However, addition of exogenous membrane-permeable derivatives of cAMP or of cyclic nucleotide phosphodiesterase inhibitors will maintain meiotic arrest (Nekola and Moor-Smith, 1975). If gonadotrophins are added to the cultures at

physiological levels, maturational arrest will be relieved (Dekel and Beers, 1978). Beers and Olsiewski (1985) have proposed a model whereby junctional communication is terminated in response to gonadotrophin, leading to cAMP depletion in the oocyte and consequently to oocyte maturation. What the mechanism of inhibition of communication might be, or indeed whether this is what actually happens *in vivo*, remains to be determined.

Once the oocyte has been released into the oviduct, the cumulus cells gradually degenerate and by the first cleavage division are no longer present. From this point up to and including the 8-cell stage all blastomeres are totipotent. The first determination/differentiation event occurs at the late 8-cell stage, at the time of compaction (Gardner and Rossant, 1976). Gap junctions do not appear until the onset of compaction, as evidenced by experiments which show that dye spread and ionic coupling are restricted to sister blastomeres up until this stage (Lo and Gilula, 1979a). In the same paper it is demonstrated that these sister blastomeres are linked by cytoplasmic bridges since horseradish peroxidase, which is too large to pass through gap junctions, will rapidly spread between sister blastomeres. At compaction several changes take place simultaneously. The blastomeres flatten and tight junctions form, isolating the inside of the cell aggregate from the outside environment. The cells become junctionally coupled (Lo and Gilula, 1979a), and the trophoblast/ICM lineages become determined. The

precise relationship between these events is not known, although it has been determined that gap junction formation is independant of compaction, and vice versa (Goodall, 1986; Kidder *et al.*, 1987). The timing of these events has prompted Lo (1985) to suggest that junctional communication might mediate the formation of an inside-outside gradient and that this gradient may provide the signalling required for specifying the trophoblast/ICM lineages.

Dye coupling persists throughout the entire embryo from the compacted 8-cell morula to the blastocyst stage. Post-implantation, this pattern of junctional communication changes so that by the end of the 6th day post-coitum, when the trophoblast cells undergo giant cell transformation, they become dye-uncoupled with one another and with the ICM (now known as the primitive ectoderm, or epiblast), although ionic coupling persists (Lo and Gilula, 1979b). In contrast, the epiblast cells remain strongly dye-coupled. As such embryos undergo further differentiation, the epiblast differentiates into the extraembryonic endoderm and the embryonic/extraembryonic ectoderm. During this time, Lo and Gilula (1979b) observed that the epiblast cells became further subdivided into a number of additional communication compartments which approximately correspond to areas within which all cells share a similar developmental fate. These observations have prompted them to speculate that the formation of communication

compartments is a developmentally significant process, enabling diverse morphogenetic gradients to be set up within adjacent fields of cells, so determining cell lineages within the early embryo.

This hypothesis is given credence by the discovery of communication compartments within a variety of other developing systems. One such system which is both well-defined and amenable to experimental manipulation is the insect imaginal disk. Imaginal disks are known to be divided into a number of discrete multicellular domains that are detectable by lineage analysis (Garcia-Bellido *et al*., 1973, 1976). These domains, referred to as developmental compartments, appear to constitute the realm within which the pattern-regulating homeotic genes are differentially expressed (Garcia-Bellido, 1975). Weir and Lo (1982; 1984) have performed an extensive analysis of patterns of dye transfer within the Drosophila imaginal wing disk. They were able to detect the presence of nine communication restriction boundaries, five of which appeared to coincide with the borders of develpmental compartments. Comparable observations have been made with the milkweed bug Oncopeltus, in which segmental borders of the integument correspond to partial communication restriction borders (Blennerhassett and Caveney, 1984), and likewise with the beetle Tenebrio (Caveney and Safranayos, 1985). Similarly, regions of restricted communication in the embryo of the mollusc Lymnaea Stagnalis, which has been

mapped by lineage studies, coincide with the borders between developmentally disparate compartments of cells both in space and in time (Serras and Van Den Biggelaar, 1987), suggesting a causal relationship between developmental and communication compartments.

Further evidence for an involvement of metabolic cooperation with developmental processes has been gained from studies involving the inhibition of metabolic cooperation in developing systems. For instance, it has been speculated that the developmental pattern abnormalities caused by application of retinoic acid to the regenerating amphibian limb could be a result of blocking junctional communication (Maden, 1982).

More convincing evidence has been presented by Warner *et al.* (1984), using antibodies raised against the 27K gap junction protein (see section 1.15). Injection of these antibodies into individual cells within Xenopus embryos at the 8-cell stage completely blocked metabolic cooperation within those cells and their progeny to the 32-cell stage, as measured by electrical coupling and dye transfer (see section 1.12), and when such embryos were allowed to develop further, gross abnormalities were observed in the progeny of the antibody-injected cells. Attempts to produce completely communication-incompetent embryos by injecting cells just before the first cleavage, or into both cells at the 2-cell stage, were unsuccessful; all such embryos died before gastrulation.

Using the same antibody preparations, Bode *et al.* (1987) and Fraser *et al.* (1987) have investigated the role of gap junction mediated intercellular communication in the mechanism of head inhibition in hydra. A series of transplantation experiments have demonstrated that a pair of developmental gradients play a central role in locating the developing head at the apical end (MacWilliams, 1983a and b). One of these gradients is termed the head activation gradient. If a hydroid is bisected anywhere along the body column, the apical end of the lower part will regenerate a head. Another way to demonstrate this property is to excise a piece of the body column and implant it in the lower part of the body column of a second animal. A fraction of these implants will develop into second heads. The ability to form a secondary axis has been found to be graded down the body column (Webster and Wolpert, 1966; MacWilliams, 1983b), so that a higher percentage of secondary axes will be formed by implants from the upper portion of the body column than from the lower portion. This is a local tissue property and is quite stable, since different regions maintain their differences upon excision and implantation at a new axial level in a second host. It is not an immutable property though, and gradually an implant will take on a head activation value characteristic of its new position (the half-life of the head activation property is approximately 36 hours). The head activator has been isolated and is an 11 amino acid

peptide (Schaller, 1973; Schaller and Gierer, 1973). The second gradient is one of head inhibition. This may be demonstrated by transplanting tissue from equivalent regions of hydroids into hosts at various positions along the body column. It is found that the closer one gets to the head, the lower is the incidence of secondary axis formation. Also, removal of the existing head results in a dramatic increase in the fraction of implants forming secondary axes (Webster, 1966; MacWilliams, 1983a). Removal of the head results in the disappearance of the head inhibition gradient with a half-life of 2-3 hours, and the inhibitory effect of the head is exerted over distances of up to 5mm. The head inhibitor has been purified (but not yet fully characterised) and has been shown to be a substance with a molecular weight of <500 daltons. This, combined with the rapidity of, and wide distribution of, the head inhibition property is consistent with the idea that it may diffuse from cell to cell via gap junctions. In order to investigate the effect of the antibodies upon the transmission of the head inhibition gradient, hydroids were bathed in a solution containing 5% DMSO at 4°C in the presence of one of the two antibodies (antibody A or antibody B). Antibody A blocked the passage of injected Lucifer yellow for 6-8 hours and antibody B blocked dye passage for 12 hours. This treatment had no obvious detrimental effect upon the health of the animals. A series of transplantation experiments were carried out upon hydroids loaded with antibodies A and B along with

control animals either left untreated, decapitated, or loaded with immunoglobulins isolated from preimmune serum. It was found that in the anti-gap junction antibody treated animals there was a marked decrease in head inhibition as compared to the intact controls, although not quite as much as in the decapitated control. These results are interpreted as providing strong support for the hypothesis that head inhibition is passed from cell-to-cell via gap junctions, and lends weight to the increasing body of evidence that gap junctions may play a central role in the transmission of developmental signals.

However, it must be borne in mind that the xenopus and hydra experiments are subject to criticism on the grounds of the specificity of the antibodies (see section 1.16), and in the absence of definitive proof that these antibodies interact exclusively with gap junctions, they cannot be regarded as absolute proofs of the involvement of metabolic cooperation in these developmental processes.

1.21 RATIONALE OF THIS PROJECT

The lack of inhibitors which interact exclusively with the gap junction protein to block metabolic cooperation has made the investigation of possible roles of junctional communication in development a difficult task.

Therefore a number of investigators have adopted a genetic approach to the problem, selecting cooperation defective EC cell lines and investigating the effect of the defect upon their *in vitro* and *in vivo* differentiation properties (see section 1.18). To date, the effects of metabolic cooperation in such variants have largely been obscured by a general decline in differentiation capacity related to factors such as the lengthy selection procedures involved, or to treatment of cultures with mutagenic agents. Therefore the evidence for an involvement of metabolic cooperation with the process of cellular determination in EC systems remains circumstantial. The main concern of this project was to repeat and refine the aforementioned selections using pluripotent embryonic stem cells as starting material, the aim being to improve the efficiency of selection and thereby to minimise as far as possible the length of time spent in culture, in the hope that this would reduce non-specific deleterious effects upon differentiation to a level whereby meaningful conclusions could be drawn as to the role of metabolic cooperation in this system.

Prompted by reports that the mutagen MNNG exerts a deleterious effect upon lens cell transdifferentiation (Clayton and Patek, 1985), and upon EC cell differentiation (see section 1.18), an investigation was carried out into the effects of three mutagens; MNNG, ICR-191 and DEO, upon ES cell differentiation. Specifically, I have compared the capacity of mutagenised

and non-mutagenised populations of the ES cell lines E14 and B2B2 to form cavitated embryoid bodies.

The aliphatic alcohols 1-heptanol and 1-octanol, which have previously been reported to be strong inhibitors of metabolic cooperation in various systems (section 1.15). In chapter 5 an investigation into the effect of heptanol and octanol upon metabolic cooperation in the ES cell line B2B2TG5 is reported. The EC cell line PTmr0 is a variant line selected for resistance to inhibition of metabolic cooperation by retinoic acid. With a view to gaining further insight into the nature of this lesion, the effect of heptanol upon metabolic cooperation in PTmr0 has been investigated.

Finally, it became clear early on in this investigation that due to the high cell numbers employed for the selections, a bulk method for the production of BRL-conditioned medium was desirable. Therefore a method has been developed for the production of BRL-conditioned medium using mass culture upon Cytodex-3 microcarriers.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL LINES

The STO mouse fibroblast line (Martin and Evans, 1975) is a thioguanine resistant and ouabain resistant derivative of a continuous line derived from embryos of strain SIM mice (Ware and Axelrod, 1972). 3T3-A31 fibroblasts (Aaronson and Todaro, 1968) are a subclone of a fibroblast line derived from 14-day embryos of strain BALB/C mice. Buffalo Rat Liver (BRL) cells derive from a clonal isolate from the liver of a five week old Buffalo rat (Coon, 1968). The origins and phenotypes of EC and ES cell lines employed in this study are summarised in table 2.1.

2.2 CHEMICALS AND MATERIALS

Chemicals were purchased from Sigma Chemical Co. (Poole, Dorset) or BDH (Poole, Dorset) unless otherwise specified. Mitomycin-C and retinoic acid were provided by Calbiochem (Bishops Stortford, England) or Sigma, and ICR-191 from Fluka A.G. (Basel, Switzerland). Phosphate-buffered saline tablets, Glasgow-modified Eagles medium concentrate, medium supplements, trypsin and chicken serum were obtained from Flow Laboratories (Irvine, Ayrshire), and Cytodex-3 microcarriers from Pharmacia (Milton Keynes, England). Calf serum came from Flow, Gibco (Paisley, Scotland), Sera-labs (Crawley,

TABLE 2.1

Origins and phenotypes of EC and ES cell lines employed in this study.

Name	Type	Origin	Phenotype	Modal Chromosome n
PC13.5	EC	clone of line PC13 derived from tumour OTT6050(1)	mec+ dif- f.ind.	41
PSA4TG12	EC	TG resistant clone from EC line SCC PSA4(2a,2b)	mec+ dif+ HPRT- f.dep.	40
PTmr0	EC	PSA4TG12	mec+(a) dif+ HPRT- f.dep.	42
B2B2	ES	ICM of strain 129/Sv/Ev mouse embryo.(3)	mec+ dif+ f.dep.	43
B2B2TG5	ES	TG resistant clone derived from B2B2.	mec+ dif+ HPRT- f.dep.	43
E14	ES	ICM of strain 129/Ola mouse embryo.(4)	mec+ dif+ f.dep.	40

Abbreviations; mec+ = metabolic cooperation competent
dif+ = differentiates spontaneously
dif- = does not differentiate spontaneously
f.ind. = feeder independant
f.dep. = feeder dependant

Footnotes; (1) Bernstine *et al.*, 1973
(2a) Slack *et al.*, 1977
(2b) Martin and Evans, 1975
(3) Robertson *et al.*, 1983
(4) Hooper *et al.*, 1987

(a) PTmr0 is a metabolic cooperation variant resistant to the inhibition of metabolic cooperation by 10^{-4}M retinoic acid (Smith *et al.*, 1986)

England), or Northumbria Biologicals (Cramlington, England). Tissue culture plasticware was obtained from Flow and from Becton Dickinson (Twickenham, England).

Rabbit polyclonal anti-serum directed against mouse alpha-fetoprotein (AFP) came from Miles Laboratories Ltd. (Stoke Poges, England), swine anti-serum directed against rabbit immunoglobulins came from Dakopatts (High Wycombe, England), and rabbit peroxidase anti-peroxidase complex and normal swine serum were provided by the Scottish Antibody Production Unit (Carluke, Scotland).

K2 Nuclear Research emulsion was obtained from Ilford. D19 developer was obtained from Kodak. Amfix fixative was obtained from Hamilton Tait (Photographic suppliers, Edinburgh).

2.3 CELL CULTURE

Cell culture was carried out either in disposable plastic tissue culture flasks or plates, or on Cytodex-3 microcarriers in a Techne microcarrier culture apparatus. Media and cells were transferred using Volac serological pipettes.

Pipettes were soaked overnight in Calgonite (100g/l), washed in water and then 0.01M HCl, rinsed thoroughly with tap water and then distilled water, oven-dried at

180°C, and sterilised in canisters by heating to 160°C before use. Glassware for use with microcarriers was first siliconised by rinsing with dimethyl-dichloro-silane, baking in a hot-air oven and washing thoroughly.

Cells were grown in Glasgow-modified Eagles medium (Stoker and McPherson, 1961) supplemented with non-essential amino acids (Proline and Serine at 0.2mM, others 0.1mM each), 1mM sodium pyruvate, 10% foetal or newborn calf serum (selected batches), and 0.1mM 2-mercaptoethanol (Oshima, 1978). This complete medium will henceforth be referred to as CMβ. Cultures were maintained at 37°C in a humidified atmosphere of 95% air, 5% CO_2. With the exception of E14, cells were routinely cultured in CMβ containing newborn calf serum. E14 cells were grown in CMβ containing foetal calf serum. Cultures were checked periodically for contamination with mycoplasma using the method of Chen (1977), modified by the use of Human fibroblasts (GM3171, Human Genetic Mutant Cell Repository, Camden, N.J.) as recipients onto which samples of test cultures were seeded. These are large cells with a high proportion of cytoplasm, making the detection of the small fluorescent points characteristic of mycoplasma infection a simple task. Any cultures suspected of being contaminated were discarded.

Stocks of PC13 and its derivatives were grown in CMβ on

plastic surfaces pre-coated with gelatin (Bernstine *et al.*, 1973). The plastic was covered with a 0.1% gelatin solution, then incubated at 4°C for at least ten minutes to allow adsorption onto the surface of the plastic. The excess gelatin was then aspirated away. All other EC and ES lines were grown either on STO feeder layers in CMβ (section 2.34) or on gelatin coated surfaces in medium conditioned by incubation with Buffalo Rat Liver (BRL) cells (section 2.35), diluted to 60% with unconditioned medium (60% BRLcm).

2.31 PASSAGING OF CELLS

Cells to be passaged were first washed twice with phosphate buffered saline (PBSA). This is an aqueous solution containing 8g/l NaCl, 0.2g/l KCl, 1.15g/l Na_2HPO_4 and 0.2g/l KH_2PO_4. They were then disaggregated with TVP (trypsin 0.25g/l, disodium EDTA 0.37g/l and chicken serum 10ml/l in PBSA). The trypsin was then neutralised with medium containing serum. Split ratios were generally of the order of 1:10.

Microcarrier cultures were disaggregated by washing the microcarriers three times with PBSA, incubating for 10 minutes with TVP (with constant agitation), then washing out detached cells by placing the microcarrier suspension in a cellector sieve (Bellco) and rinsing thoroughly with CMβ.

2.32 MICROCARRIER CULTURE

Microcarriers were hydrated at room temperature by incubation for at least 3 hours with approximately 25ml PBSA per gram of microcarriers. As a wetting aid, 2 or 3 drops of Tween-80 were dissolved in the PBSA prior to the introduction of microcarriers. They were then washed in three changes of PBSA, autoclaved, and stored at 4°C until needed. For use, they were washed twice with CMβ and then poured into a Techne microcarrier culture vessel. Cells were introduced at a density of 5 x 10^7 cells/g microcarriers and attachment was achieved by intermittent stirring for 3 hours (2 minutes on, 15 minutes off at 20rpm), followed by continous stirring at 30rpm. Cultures were fed by allowing the microcarriers to settle onto the bottom of the vessel, discarding the medium, and pouring on fresh medium.

2.33 FREEZING AND THAWING OF CELLS

Frozen stocks of cells were prepared by suspending cells in CMβ supplemented with 10% (v/v) DMSO, pelleting cells by centrifugation, then re-suspending in fresh CMβ/10% DMSO at a density of at least 4 x 10^6 cells/ml. Aliquots (0.5-1ml) of this suspension were then transferred to serum test tubes and stored overnight in insulated racks

in a -70°C freezer, such that they were cooled at a rate of approximately 1°C/minute. The tubes of cells were then transferred to the vapour above liquid nitrogen. In contrast to the freezing process, cells were thawed rapidly by immersing the vials in a 37°C water bath (being careful not to allow water to come into contact with the seal, as a precaution against contamination), and agitating gently. The cells were then re-suspended in 10mls of medium. DMSO was removed by spinning the cells and re-suspending in fresh medium.

2.34 PREPARATION OF FEEDER LAYERS

Feeder layers were prepared from STO cells unless otherwise specified. Confluent cultures were treated with medium containing 0.01mg/ml mitomycin-C for 2 hours at 37°C (Martin and Evans, 1975). The cells were washed three times with PBSA, trypsinised and seeded out at a density of 4×10^4 cells/cm^2. The integrity of STO feeder layers depends critically on the condition of the STO cells on mitomycin treatment. For the formation of good feeder layers it is important to use STO cells which are subject to density dependant growth inhibition. High passage STO stocks (above passage 30), were discarded. Feeder layers were considered to be suitable for use for approximately one week after mitomycin treatment.

For ouabain rescue experiments (section 2.71) STO feeder

layers were prepared in situ. Cultures were established at a density of 4 x 10^4 cells/cm^2 and exposed to mitomycin-C as above. The monolayers were then washed three times with PBSA and incubated in CMβfor 3 hours. EC or ES cells were then inoculated in fresh medium. 3T3-A31 feeder layers were likewise prepared in situ.

2.35 PREPARATION OF CONDITIONED MEDIUM

Medium was conditioned by incubating CMβ upon a confluent mono-layer of BRL cells, either in 175cm^2 tissue culture flasks or in microcarrier culture. For flask conditioning, 30ml/flask was employed. For microcarrier conditioning, 3.8g microcarriers per litre of CMβ was adopted as standard (see chapter 6). In both cases, medium was incubated for three days then pipetted or decanted off, filtered with a 0.8um filter and diluted to 60% (v/v) with fresh CMβ to yield working strength 60% BRLcm. Cultures of BRL cells were maintained for up to a month before being discarded. No deleterious effects upon the quality of the medium were observed over this time-scale. BRLcm was routinely stored frozen at -20°C until required for use. Medium kept in storage for greater than 6 months was discarded.

2.36 ESTABLISHMENT OF CLONAL LINES

Clonal lines were established by dilution plating of single cell suspensions into multi-well plates. Only where a single colony grew up in a well, was this trypsinised and expanded into a cell line.

2.4 MUTAGENESIS

The LD_{50} value for ICR-191 was initially determined by plating 500 B2B2TG5 cells each onto ten gelatinised 60mm plates and on the following day introducing ICR-191 in fresh 60% BRLcm at a range of concentrations. After 24 hours incubation, the mutagen was removed and plates were washed twice with CMβ. The plates were maintained for 7 days in culture, then fixed and stained with Leishmans' stain, and colonies counted (table 2.2, figure 2.1). The LD_{50} value was 0.35μg/ml.

In the light of the observation that this concentration resulted in a lower than expected kill in the mutagenesis step prior to the initial round of thioguanine selection, a subsequent test was performed at higher cell density. This time ICR-191 at seven concentrations ranging from 0 to 2.5μg/ml was applied to duplicate cultures of B2B2TG5α cells at a density of 7.5×10^4 cells/cm² on 60mm plates (ICR-191 was introduced immediately upon seeding of cells). After 24 hours the plates were washed with two

TABLE 2.2

The toxicity of ICR-191 to B2B2TG5 cells.

conc. ICR-191 (μg/ml)	mean no. of colonies	% survival
0	228	100
0.1	179.5	78
0.2	147	65
0.5	88.5	39
1	10.5	5

Results are quoted as the mean of two determinations; initial inoculum 500 cells per 60mm plate.

FIGURE 2.1

Survival vs concentration plot for ICR-191 upon B2B2TG5 cells seeded at 500 cells per 60mm plate

Abscissa = concentration of ICR-191 (μg/ml)

Ordinate = percentage of surviving colonies relative to the control

ICR-191 toxicity
100
80
60
40
20
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1
B

changes of fresh CMβ then re-fed with 60% BRLcm and left for another 24 hours. Then the cultures were trypsinised and cells re-seeded onto fresh plates (this step was intended to remove dead and dying cells). Twenty-four hours later, the cultures were again trypsinised and cell counts performed using a haemocytometer. The concentration at which approximately 50% of cells were killed was 1μg/ml (table 2.3, figure 2.2). Cells for the initial round of selection were mutagenised with 0.35μg/ml ICR-191. For the third round of selection, cells were mutagenised with 0.5μg/ml ICR-191. For the HAT selections, cells were mutagenised with 1.5μg/ml ICR-191. A cell density of 7.5 x 10^4 cells/cm^2 was used throughout, and following 24 hours incubation with ICR-191, cultures were allowed at least 48 hours recovery time prior to feeding into selections (see chapter 3 for details).

For the investigation into the effects of mutagens upon differentiation capacity, toxicity tests were performed for ICR-191, DEO, and MNNG upon E14 and B2B2 cells (chapter 4). The latter method was used, with the modification that DEO was applied for 1 hour and MNNG was applied for 2 hours. Also, the cell density in the E14 test was 2 x 10^4 cells/cm^2. As DEO and MNNG were dissolved as 10mM stock solutions in DMSO, appropriate amounts of DMSO were added to control cultures for the duration of mutagenesis. ICR-191 was dissolved in water to make a 100μg/ml stock solution.

TABLE 2.3

The toxicity of ICR-191 to B2B2TG5 cells

conc. ICR-191 (μg/ml)	mean no. of cells	% survival
0	1.3×10^7	100
0.5	9.4×10^6	72
1	5.5×10^6	45
1.5	4.1×10^6	31.5
2	3.6×10^3	3
2.5	0	0

Results are quoted as the mean of two determinations; initial inoculum 2×10^6 cells per 60mm plate.

FIGURE 2.2

Survival vs concentration plot for ICR-191 upon B2B2TG5 cells seeded at 7.5×10^4 cells per cm^2

Abscissa = concentration of ICR-191 (μg/ml)

Ordinate = percentage of surviving cells relative to the control

ICR-191 toxicity (2nd det.)
180
135
90
45
0
0
0.5
1
1.5
2
2.5
B

2.42 CALCULATION OF MUTATION FREQUENCIES

Mutation frequencies were determined by selecting thioguanine resistant ($HPRT^-$) mutants from mutagenised cultures of E14 cells. Three cultures of 2.5×10^7 cells were mutagenised at a cell density of 2×10^4 cells/cm^2 with 0.5µg/ml ICR-191, 0.05mM DEO and 0.01mM MNNG. Following a recovery period of 48 hours, cultures were trypsinised and cells seeded at a density of 7×10^3/cm^2 on 100mm plates. After overnight incubation, cultures were fed with medium containing 6-thioguanine at a concentration of 10µg/ml. Cultures were maintained in thioguanine medium for ten days, being re-fed every other day, followed by unmodified medium for a further seven days. The plates were then fixed and stained with Leishmans' stain and colonies counted. The induced mutation frequency was calculated and compared with the natural mutation frequency in an untreated control culture maintained in parallel (see chapter 4).

2.5 TOXICITY OF 1-HEPTANOL AND 1-OCTANOL

B2B2TG5α cells were plated onto gelatinised 60mm plates at 500 cells/plate, incubated overnight and then the appropriate quantity of heptanol or octanol was added. The test medium was renewed daily for three days, then replaced with unmodified 60% BRLcm. Plates were

maintained for a further four days, then fixed and stained with Leishmans' stain, and colonies counted. All plates were set up in duplicate, and two determinations were performed. Control cultures were supplemented with 0.1% (v/v) absolute ethanol for the duration of heptanol/octanol treatment, as heptanol and octanol were dissolved as either 3M, 1M or 100mM stock solutions in absolute ethanol, and this represented the highest concentration of ethanol present during any toxicity test.

2.6 INDUCTION AND ANALYSIS OF ES CELL DIFFERENTIATION

2.61 INDUCTION OF DIFFERENTIATION BY FORMATION OF AGGREGATES

Cells were seeded at high density (2.5 x 10^6 cells per 60mm dish) in Eagles medium supplemented with 10% newborn calf serum and β-mercaptoethanol but without non-essential amino acids and sodium pyruvate (EC10 medium). Cultures were fed as necessary until nests of cells had formed (about three days). Aggregates were detached by blowing fresh medium across the surface of the dish, transferred to tissue culture dishes coated with 2% agarose (to which they could not attach), and grown for up to 25 days in EC10 medium.

After suspension culture, embryoid bodies were

transferred to universal containers and allowed to sediment under gravity. They were fixed in Bouin's fluid then spun down in molten agar. The agar was allowed to set, and the embryoid bodies were subjected to routine histological processing and embedded in paraffin wax. 4-5μm sections were cut and then either stained with Mayer's Haematoxylin and Eosin, or immunohistochemically stained using the AFP/PAS stain as described in section 2.62.

2.62 AFP/PAS/HAEMATOXYLIN STAIN

In order to differentiate visceral and parietal endoderm, embryoid body sections were stained by a combination of immunoperoxidase staining for alpha-fetoprotein (AFP) and periodic acid Schiff (PAS) staining with a light haematoxylin counterstain.

After dewaxing in xylene (10 minutes), sections were rehydrated through descending concentrations of ethanol to distilled water. They were then washed in two changes of TBS (5 minutes). TBS is composed of 0.6g of Tris (Trizma base, obtained from Sigma) dissolved in 1 litre of a solution of 8.5g/l NaCl and adjusted to pH 7.6 with HCl. Sections were incubated for 5 minutes in 20% (v/v) normal swine serum in TBS (NSS/TBS) and drained before being incubated with the primary antibody, polyclonal rabbit anti-mouse alpha-fetoprotein, at a dilution of

1:50 in NSS/TBS, for 30 minutes. After this period they were again washed with TBS (3 minutes each) and incubated for 5 minutes with NSS/TBS. They were drained before being incubated with the secondary antibody, swine anti-serum directed against rabbit immunoglobulins, at 1:30 dilution in NSS/TBS, for 30 minutes. This was followed by a repetition of washing as above, then incubation with the tertiary antibody, rabbit peroxidase anti-peroxidase complex diluted to 1:100 in NSS/TBS, for 30 minutes. Sections were again washed with TBS (2 changes, at least 5 minutes each), then freshly prepared DAB solution was applied for at least 5 minutes, by which time all AFP-positive regions were adequately stained, and then the reaction was stopped with tap water. DAB solution was prepared by dissolving 5mg of diaminobenzidine in a solution of 3mg imidazole in 5ml TBS (adjusted to pH 7.6) and adding 0.1ml of a 1% (v/v) solution of 100-volume H_2O_2 in water immediately prior to use.

After AFP immunoperoxidase staining sections were incubated with 1% periodic acid for 5 minutes, and well rinsed in tap water, before incubation with Schiff reagent for 7 minutes. They were again washed in tap water for 10 minutes before counterstaining lightly with Mayer's haematoxylin. Schiff reagent stains regions of basement membrane bright pink. Sections were dehydrated and mounted in the conventional fashion.

The criteria used for scoring embryoid body

differentiation were as follows:

Category A = undifferentiated aggregates consisting wholly of ES-like cells

Category B = endoderm present in aggregates, but no cavitation

Category C = cavitated embryoid body

Classes B and C were further subdivided by scoring AFP-positive and AFP-negative embryoid bodies. Non-cavitated embryoid bodies were only placed in category B if there was definite morphological evidence of endoderm. The presence of non-specific AFP-positive regions or Schiff-staining regions was not considered to be sufficient evidence alone.

2.63 DIFFERENTIATION OF EMBRYOID BODY OUTGROWTHS

Outgrowths were formed by allowing six-day embryoid bodies to settle onto Thermanox coverslips (Lux) in complete medium. The cultures were incubated for two weeks, being re-fed as necessary. The coverslips were picked up and immersed in Bouin's fluid, embedded in paraffin wax, and after routine histological processing, sectioned and stained with Mayer's haematoxylin and eosin.

2.64 CELL COLONY DIFFERENTIATION IN BRL CONDITIONED MEDIUM

In the first differentiation test, cells maintained in flask conditioned BRLcm were plated into 100mm plates at 3000 cells per plate, in the appropriate microcarrier conditioned BRLcm. After 10 days incubation (medium changed every other day), plates were fixed and stained with Leishmans' stain and colony counts and areas computed using the IBAS-2 image analyser. The differentiation state of colonies was judged qualitatively on the basis of morphology. For the second test, cells maintained in flask conditioned 60% BRLcm were plated at 1000 cells per 60mm plate into the appropriate microcarrier conditioned 60% BRLcm, and incubated for 10 days, being re-fed every other day. Then plates were fixed and stained as above and colonies were counted and assessed for differentiation on the basis of morphology under the dissecting microscope. A third test was also carried out. This time B2B2 cells were employed and plated out at 500 cells/60mm plate. Aside from this, the procedure was identical to the above method.

2.7 QUANTIFICATION OF METABOLIC COOPERATION

2.71 RESCUE FROM OUABAIN TOXICITY

Cells were seeded at 10^3 per 60mm plate on STO feeders. After overnight incubation half the dishes were treated with medium containing 3mM ouabain and half with unmodified medium. Three days later, all the dishes were returned to normal medium for 5-7 more days growth. Plates were than fixed and stained with Leishmans' stain for colony counting.

For testing the effect of heptanol and octanol upon metabolic cooperation, the respective alcohols were added to plates at the same time as ouabain, and their presence maintained for the duration of ouabain treatment.

The index of cooperation is defined as the mean number of colonies which grow up in the presence of ouabain, divided by the mean number in its absence, divided again by the corresponding ratio for a control population. A high ratio corresponds to rescue of a large proportion of cells, and a low ratio to poor rescue (see section 1.17 for details). Cells cooperating as well as the control should give a ratio of 1.

2.72 URIDINE NUCLEOTIDE TRANSFER

2×10^5 cells were seeded onto each of a number of gelatinised 30mm tissue culture plates or slide flasks (Nunc). Some of these plates were to serve as donor cells, others as recipients. They were then incubated overnight. On the following day, donors were incubated in medium containing 10μCi/ml ^{3}H-uridine for 3 hours to allow incorporation into the soluble nucleotide pool. Then they were washed three times with PBSA, trypsinised and seeded onto recipients at a 1:10 split ratio. Donor-recipient mixtures were incubated for 3 hours, followed by three washes with PBSA and fixation by inversion over tissues soaked with freshly prepared ice-cold methanol/glacial acetic acid (3:1 v/v) for at least 10 minutes. The fixed preparations were washed twice with ice-cold 5% TCA to extract unincorporated nucleotides, washed overnight with tap water and air-dried at 37°C for at least 5 hours.

K2 Nuclear Research emulsion was melted at 50°C under a red safe-light, and mixed with water in a ratio of emulsion:water of 1:1.5 (v/v). Plates were coated with emulsion by pipetting a small volume of this mixture onto a dish and transferring the excess to other plates. Plates were dried for at least 1 hour in a stream of cool air in a dark box.

The plates were stored at 4°C in the presence of silica gel in dark airtight tins. They were exposed for

approximately three days, warmed to room temperature, then developed in Kodak D19 developer for 3 minutes at room temperature, washed once with water, and fixed for 5 minutes in Amfix. The autoradiographs were then washed for 10 minutes in running water, air-dried and lightly stained with Leishmans'. The preparations were again air-dried, and examined directly at 1000x under oil immersion.

Where applicable, recipient cells were treated with various concentrations of heptanol, octanol, or retinoic acid for various lengths of time. See chapter 5 for details.

The dishes were scored by counting grains over 100 recipients in contact with donors and 100 isolated recipients per dish. The isolated recipients scored were selected as those closest to the contacting recipients scored, where there was clearly no contact either directly or through chains of cells. The proportion of contacts showing transfer was estimated by a method devised by Smith *et al*. (1986), and estimates of the mean proportion of positive contacts and the mean ouabain rescue index for each cell line, standardised for variation between experiments, were determined by logistic regression analysis (McCullagh and Nelder, 1983) using the GLIM package made available via the Edinburgh Multi-Access System by the Edinburgh Regional Computing Centre. The statistical significance of the difference

between any two estimates was assessed by computing an F-statistic from the scaled deviances of a complete regression model and a reduced model in which the two estimates were constrained to be identical (McCullagh and Nelder, 1983). In the case of ouabain rescue, those experiments used to select clones for further analysis were omitted from the statistical analysis. All statistical analyses were carried out by Dr. M.L. Hooper.

2.8 KARYOLOGY

Log-phase cultures were treated with 100μg/ ml colchicine for 2 hours at 37°C. Cells were trypsinised, spun down, and resuspended gently in 0.075M KCl. After a 4 minute incubation at room temperature, the cells were spun down again. They were then fixed by drop-wise addition of ice-cold methanol: glacial acetic acid (3:1 v/v) and left for at least 30 minutes, spun down again, and resuspended in fresh fix. Drop preparations were made onto acid-cleaned microscope slides. The chromosomes were than stained with either Giemsa or Leishmans' stain, and chromosomes in unbroken spreads counted.

2.9 SELECTION OF VARIANT CELL LINES

2.91 THE THIOGUANINE KISS-OF-DEATH: OPTIMISATION OF SELECTION CONDITIONS

In order to optimise selective conditions with respect to the ratio of donor cells to recipient cells, and to discover the most suitable cell type for the various rounds of selection, mock selections were carried out using PC13.5 and B2B2.2 (a clonal derivative of B2B2). B2B2TG5 cells were used as recipients and were mixed with either of the donor cell types at a ratio of 3 donors:1 recipient or 6 donors:1 recipient. These cell mixtures were seeded at a density of 10^5 cells/cm^2 onto 100mm tissue culture plates (three plates per test) and allowed to attach overnight. Medium containing 10μg/ml 6-thioguanine was then added to the plates, and cultures were maintained in this selective medium for five days, being re-fed every other day. At the end of this period, the cultures were washed twice with PBSA then fed with unmodified medium, in which they were maintained for a further ten days. Plates were then fixed and stained with Leishmans' stain and colonies counted.

To determine the effect of re-seeding cultures with fresh donor cells during the selection period, two sets of plates having B2B2.2 as donor were treated initially for two days with selective medium. They were then washed twice with PBSA and re-seeded with fresh B2B2.2 cells in

unmodified medium (the number of cells seeded was identical to that used for the initial inoculation). After overnight incubation, selective medium was re-applied and the experiment allowed to proceed as above.

2.92 THE THIOGUANINE KISS-OF-DEATH SELECTION

Mixtures of donor cells and recipient cells were seeded onto tissue culture flasks or plates at an overall density of 10^5 cells/cm^2 and allowed to attach overnight. On the following day medium supplemented with 10µg/ml 6-thioguanine was applied and cultures were maintained in this selective medium for at least five days, the medium being renewed every other day. After selection, cultures were washed twice with PBSA and fed with unmodified medium. They were then maintained until either sufficient survivors were present to allow harvesting or until colonies were large enough to be picked off. Cell lines thus obtained were then expanded and assayed for metabolic cooperation.

The only exception to this general method was the initial round of selection which was performed upon microcarriers at a cell density of 5×10^4 cells/cm^2.

For details of individual rounds of selection see chapter 3.

2.93 THE HAT KISS-OF-LIFE: RECONSTRUCTION SELECTIONS

1P9 and B2B2TG5α cells were seeded onto 3T3-A31 feeder layers at 1000 cells/60mm plate. Also two sets of plates were set up in which 1P9 cells were seeded onto 3T3-A31 feeder layers at cell densities between 8 x 10^3 and 1.2 x 10^5 cells/cm^2. To one of these sets 1000 B2B2TG5α cells were added per plate. After overnight incubation, HAT medium was applied to the cultures. HAT medium consisted of 60% BRLcm supplemented with 10^{-2}M hypoxanthine, 8 x 10^{-7}M aminopterin and 2 x 10^{-5}M thymidine (Slack *et al.*, 1978). Cultures were maintained in this for three days, then for two days in medium supplemented with 10^{-2}M hypoxanthine alone. The medium was then exchanged for unmodified medium and cultures maintained for a further seven days, when they were fixed and stained with Leishmans' stain and colonies counted. HAT-free control cultures received medium containing 10^{-2}M hypoxanthine.

2.94 THE HAT KISS-OF-LIFE SELECTION

Cells were seeded onto 3T3-A31 feeder layers in 100mm tissue culture plates at a density of 6 x 10^4 cells/cm^2 and incubated overnight before being fed with HAT medium as above. After three days, the HAT medium was replaced with medium supplemented with 10^{-2}M hypoxanthine and cultures were maintained in this for a further two days. This was then replaced with unmodified medium and

cultures maintained until colonies were sufficiently large to be picked off and expanded into cell lines for characterisation.

For details of individual selections see chapter 3.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF VARIANT CELL LINES WITH ALTERED METABOLIC COOPERATION PHENOTYPES

3.1 INTRODUCTION

In order to select variant cell lines with reduced ability to communicate via gap junctions I have employed the thioguanine "kiss-of-death" technique, as used to isolate the lines R5/3 (Slack *et al.*, 1978) and PT2md1 (Smith *et al.*, 1986) - see section 1.18.

The cell line used for this study was the ES line B2B2, a pluripotent cell line which is capable of extensive differentiation *in vitro* and which can contribute to chimaeric animals with high efficiency (Bradley and Robertson, 1986). An HPRT$^-$ derivative of B2B2, B2B2TG5, was available in our laboratory and from this I have derived clones B2B2TG5α, β, δ, and Γ. B2B2TG5α gave the highest index of cooperation by ouabain rescue (figure 1.2, sections 1.12 and 2.71, and table 3.1) and proved capable of extensive differentiation *in vitro* (table 3.2), and so was chosen to form the starting material for selection.

While kiss-of-death selection is effective, it is not highly efficient and multiple rounds of selection are generally required to isolate metabolic cooperation defective variants. As it is known that prolonged culture can have a deleterious effect upon the developmental capacity of EC and ES cells, a multi-step selection is undesirable and I have introduced several modifications to the technique designed to reduce

TABLE 3.1

Ouabain rescue data; B2B2TG5 clones

clone	ouabain	no. of colonies	mean	ratio (+:-)
α	-	137,125	131	0.73
	+	77,115	96	
β	-	94,115	104.5	0.63
	+	81,51	66	
Γ	-	150,161	155.5	0.69
	+	102,113	107.5	
δ	-	253,241	247	0.48
	+	115,122	118.5	

This table refers to colony counts of duplicate cultures of B2B2TG5 clones seeded onto STO feeder layers at 500 cells/60mm plate in the presence of (+), and absence of (-) 3mM ouabain. The index of cooperation is not applicable here, since there was no control from which to derive this parameter. Rather, the ratios of survivors in the presence of ouabain to survivors in its absence are tabulated. The higher the ratio, the greater the extent of metabolic cooperation (see section 2.71).

TABLE 3.2

B2B2TG5 and B2B2TG5α embryoid bodies after 6 days in suspension.

Cell line	Number of embryoid bodies in category A	B	C	% of embryoid bodies with endoderm	% of cavitated embryoid bodies
B2B2TG5	0	29	48	100	62
B2B2TG5α	0	34	47	100	58

Embryoid body categories A, B and C are defined in section 2.62

selection times to a minimum. To this end, reconstruction selections were carried out in an attempt to optimise selective conditions (table 3.3). The general strategy was to perform an initial enrichment step calculated to give maximum recovery of variants from a large mutagenised population, followed by one or more highly stringent step(s) calculated to eliminate all but poorly cooperating cells with high efficiency.

Selection stringency depends upon the frequency with which recipient ($HPRT^-$) cells form junctional contacts with donor ($HPRT^+$) cells, and this is dependant upon several factors. The cell types used are important. In view of evidence that EC cells generally communicate with other EC cells rather more readily than with other cell types (section 1.18), the EC line PC13.5 and the ES line B2B2.2 (a clonal derivative of B2B2) were employed as donors. The overall cell density is obviously a critical factor. Previous work (Smith, T.A., 1984) has established that a density of 10^5 cells/cm^2 is sufficient to ensure a high kill of recipient cells under standard selective conditions and, with the exception of the initial enrichment step, this density was used throughout. Another factor involved is the ratio of donors: recipients. Although a ratio of 1:1 should be sufficient to ensure that all recipients make contact with at least one donor cell, in practice I have found that this is not adequate for efficient selection, especially with B2B2.2 as donor. Hence the ratio of donors:recipients in my

TABLE 3.3

Optimisation of selective conditions; the effect of the donor:recipient ratio upon selection stringency.

(mean of three determinations on 100mm plates)

Recipients; B2B2TG5
Cell density; 10^5 cells/cm^2

Donors	Ratio (D:R)	Re-seeding (2 days)	Mean no. of colonies per dish
PC13.5	3:1	-	<1
	6:1	-	<1
B2B2.2	3:1	-	67
	6:1	-	17
B2B2.2	3:1	+	<1
	6:1	+	1

selections was always 3:1 or higher. Reconstruction selections indicated that this ratio was adequate for stringent selection in the case of PC13.5 donors but surprisingly not with B2B2.2, which performed poorly even in 6:1 excess (table 3.3). This possibly reflects the fact that B2B2.2 is less easily broken up into a single-cell suspension upon trypsinisation than PC13.5. Clearly, if large clusters of donor cells persist after trypsinisation, the effective ratio of donors:recipients is significantly reduced. The kill of recipient cells can however be considerably increased by re-seeding cultures with B2B2.2 after three days of selection (table 3.3). This strategy was later employed with PC13.5 for highly stringent selection steps, and although no assay of this kind was performed during optimisation with PC13.5, it is reasonable to assume that it had the same effect as with B2B2.2.

Bearing the results of the reconstructions in mind, B2B2.2 at a donor:recipient ratio of 3:1 was used as donor for the initial enrichment step, followed by stringent rounds of selection with PC13.5 as donor. The precise details of individual rounds of selection are described below. Possibly the most significant overall improvement to the selection procedure has been the replacement of the feeder layer by BRL-conditioned medium (section 1.6 and chapter 6). Feeder cells have hampered previous selections of this kind partly because the logistics of maintaining large populations of feeders

make large-scale selections very difficult to manage. More seriously, metabolic cooperation between donor cells and feeder cells and the consequent passage of toxic nucleotides into the feeders causes them to detach from the surface. This necessitates regular re-seeding with fresh feeders and introduces the possibility that their ability to maintain an undifferentiated population of stem cells may be impaired. This could introduce selective pressure for feeder independence and a concomitant reduction in the developmental potential of those cells which survive selection, although characterisation of several clones which had been exposed to selection but which cooperated normally (Smith, Box and Hooper, 1986) indicates that this is not a serious problem.

As previously mentioned, my selection has been performed in three stages. First, an enrichment step was performed involving mild selective pressure exerted upon a very large mutagenised population of cells seeded onto microcarriers. This was intended to allow the majority of poorly cooperating cells extant within the population to survive and multiply while substantially reducing the proportion of wild type cells.

Second, a stringent selection was imposed upon a smaller population of the pooled survivors from which a small number of surviving colonies were pooled and expanded for ouabain rescue assay. These were subsequently cloned and

a clone which performed poorly in the ouabain rescue assay, 1.23, was chosen for further selection.

The third stage was a stringent selection imposed upon a mutagenised population of 1.23 cells from which a number of surviving cell colonies were recovered and expanded for assay. One poorly cooperating colony was cloned to yield the metabolic cooperation deficient line 1P9.

A subsequent round of selection upon the pooled survivors of the third round yielded the metabolic cooperation deficient line 2P2[1].

A three-fold excess of donors over recipients was used in all but one selection (for the derivation of 2P2[1] a seven-fold excess of donors was employed). Also, for the second round of selection and for the selection leading to 2P2[1], cultures were re-seeded with fresh donors after three days in selective medium, and selection resumed after overnight incubation in non-selective medium.

The mutagen employed throughout was the half-acridine frame-shift mutagen ICR-191. The reason for its use rather than the more commonly employed mutagen MNNG was that MNNG, is a base substitution mutagen, the effect of which is frequently to exchange one amino acid in the protein product of an affected gene for another. This may give rise to minor modifications of the activity of an

affected protein rather than to its complete inactivation. Frame-shifts, however, almost exclusively result in complete inactivation of the product of any affected gene. A deletion mutagen was not employed as it would have rendered the subsequent isolation of normally cooperating revertants impossible. Also, it has been reported that MNNG may have had a deleterious effect upon the differentiation capacity of PT2md1 and its derivatives (Smith *et al.*, 1986) , and a deleterious epigenetic effect of MNNG has been observed with regard to chick lens cell transdifferentiation (Clayton and Patek, 1985) (see chapter 4).

Mutagenesis was performed at two points during the selection procedure; upon the parental population of B2B2TG5α prior to the initial round of selection, and upon the intermediate variant 1.23 prior to the third round. This was based on the assumption that in a diploid system more than one mutation would be required to create a completely metabolic cooperation deficient cell line. In practice the metabolic cooperation deficient lines 1P9 and 2P2[1] are clearly capable of cell-to-cell communication, albeit at a lower level than their predecessor 1.23.

3.2 ISOLATION OF 1.23 FROM B2B2TG5α

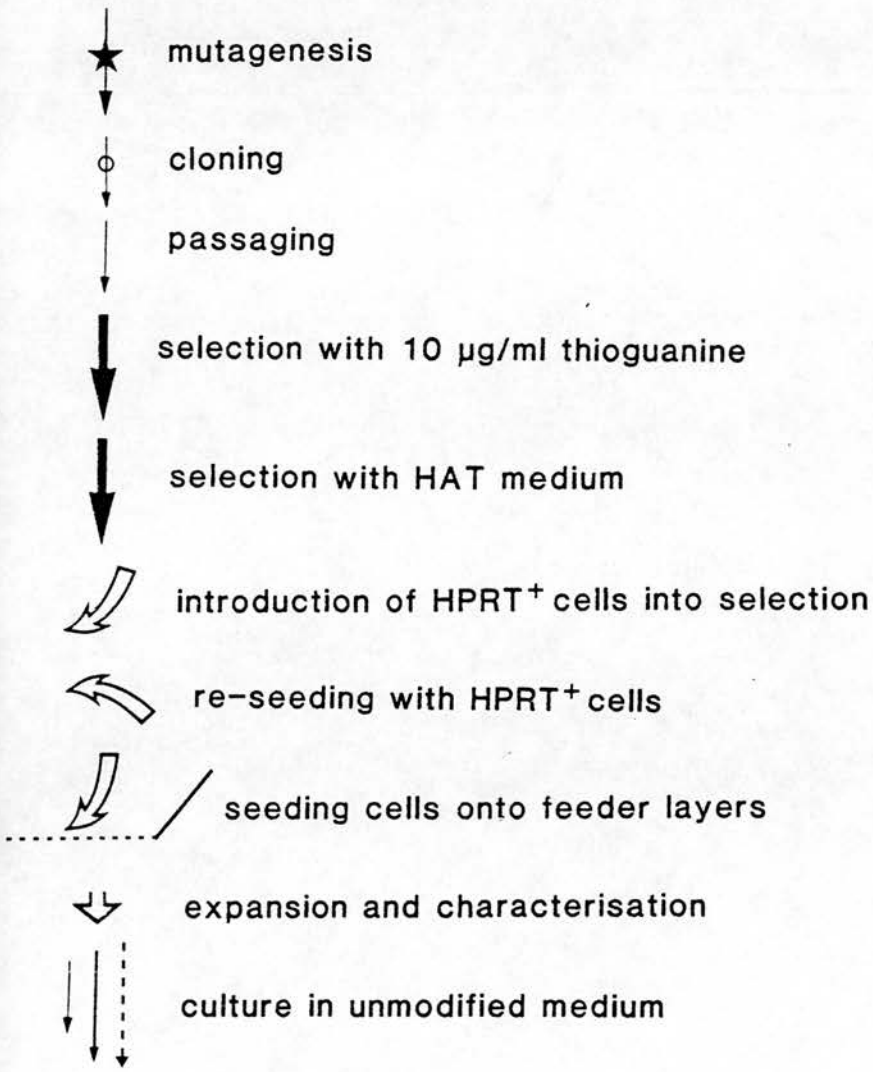
The following selections are represented schematically in figures 3.1 to 3.4. 10^8 B2B2TG5α cells were seeded into eight 175cm² tissue culture flasks in medium containing 0.35μg/ml ICR-191, which was replaced with unmodified medium after 24 hours. This treatment resulted in the death of approximately 20% of cells (see section 2.41 for kill curves). After four days recovery time the cells were trypsinised and 9 x 10^7 cells were seeded onto 1.25g of Cytodex-3 microcarriers (section 2.32) along with 2.7 x 10^8 B2B2.2 to give a starting cell density of 5 x 10^4 cells/cm². After overnight incubation, cultures were re-fed with medium containing 10μg/ml thioguanine (section 2.92) and selective conditions maintained for four days, after which the microcarriers were washed twice and re-suspended in unmodified medium. Ten days later the survivors were harvested and seeded into one 175cm² tissue culture flask.

The population was expanded to 3 x 10^7 cells and then mixed with 9 x 10^7 PC13.5 cells and seeded onto nineteen x 100mm tissue culture plates. On the following day selective medium was applied and selective conditions maintained for two days, when the plates were washed and re-seeded with 9 x 10^7 PC13.5 cells. After overnight incubation, selective conditions were re-applied and maintained for 5 days. The plates were maintained for a

Figure 3.1

Schematic representation of the selection of 1.23 from B2B2TG5 by thioguanine "kiss-of-death"

The key to the symbols used for this figure and figures 3.2, 3.3 and 3.4 is as follows:



Selection leading to 1.23

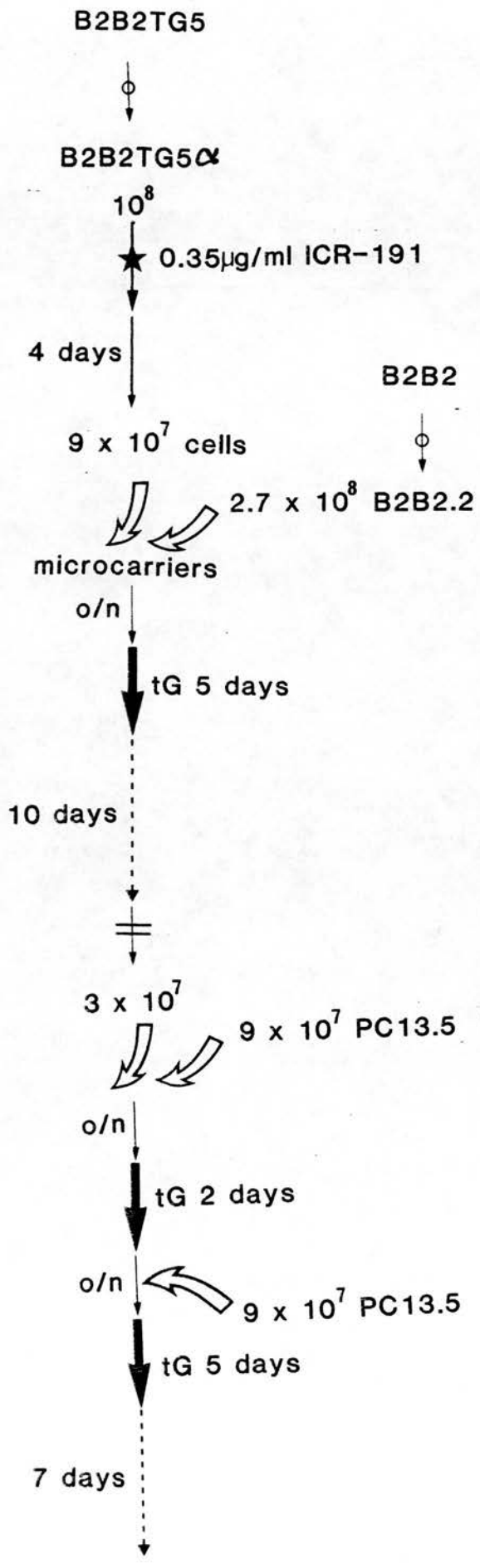


Figure 3.2

Schematic representation of the selection of 1P9 and 2P2[1] from 1.23 by thioguanine "kiss-of-death"

Selection upon 1.23

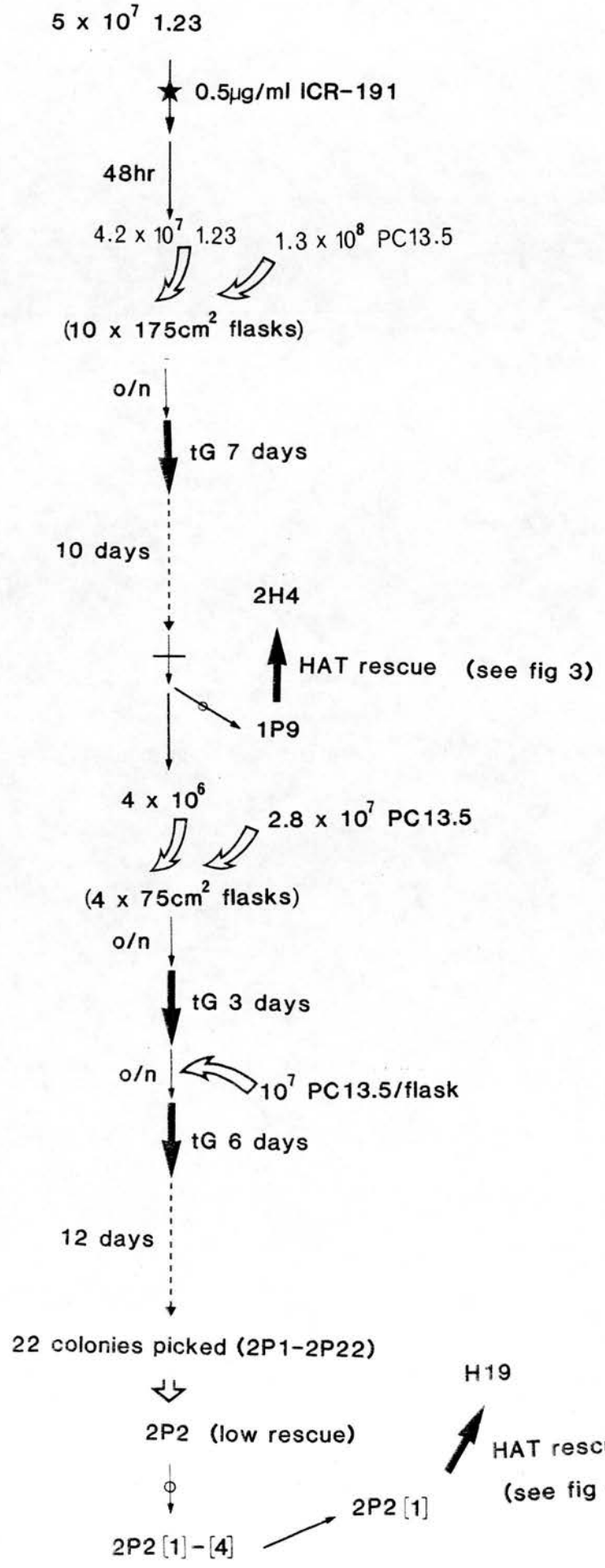


Figure 3.3

Schematic representation of the selection of 2H4 from 1P9 by HAT "kiss-of-life"

HAT rescue upon 1P9

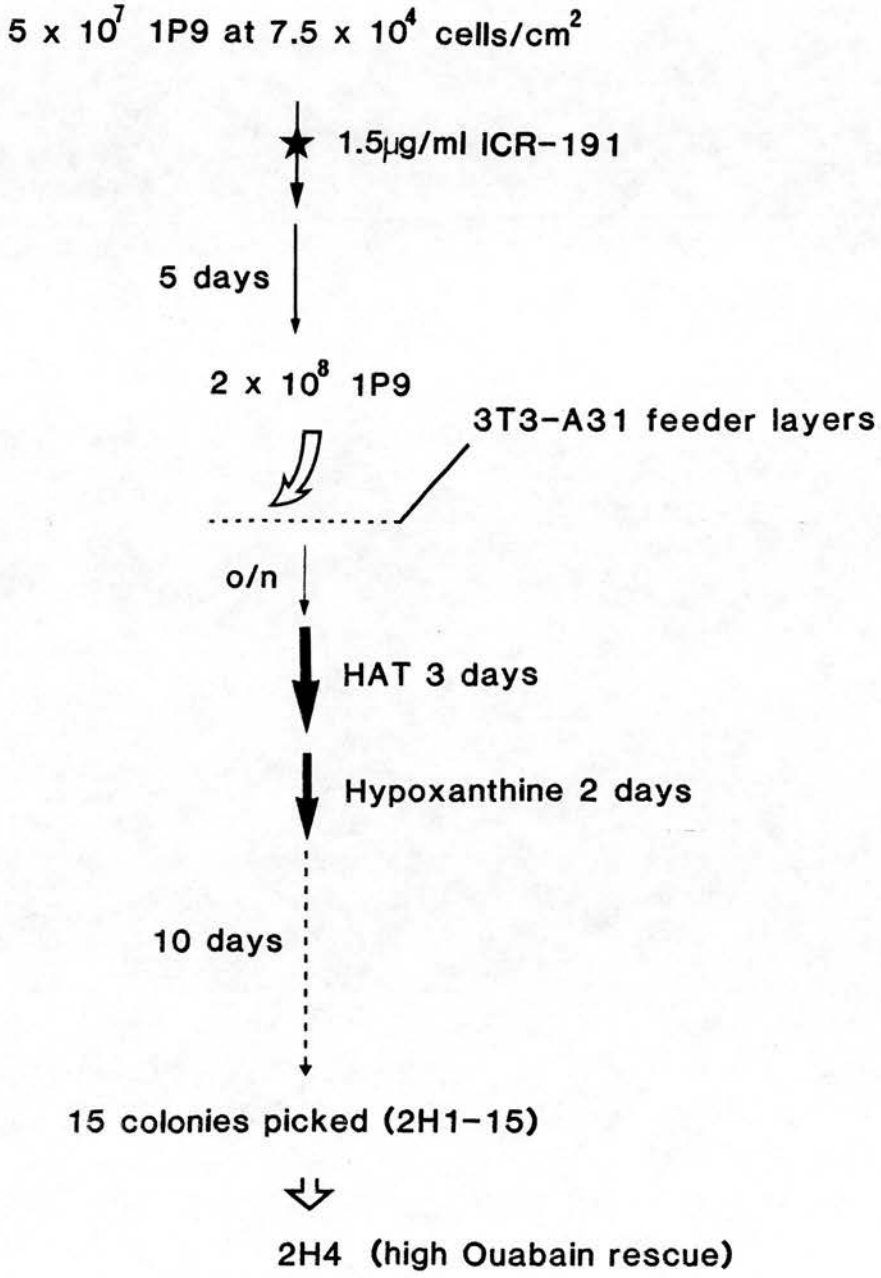
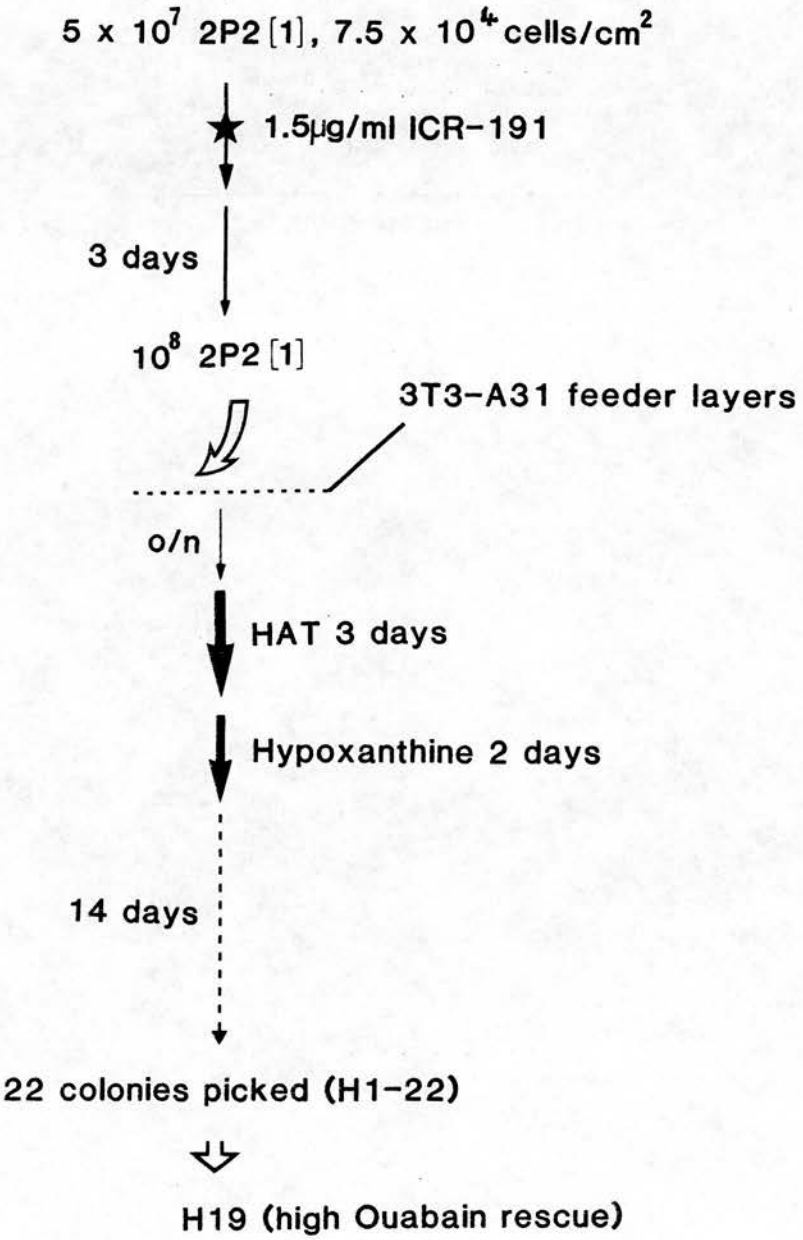


Figure 3.4

Schematic representation of the selection of H19 from 2P2[1] by HAT "kiss-of-life"

HAT rescue upon 2P2 [1]



further 7 days in unmodified medium, by which time colonies had become visible upon some of the plates. Seven colonies were picked but unfortunately four were subsequently lost due to contamination. The remaining three were pooled, expanded and cloned. Two of the resultant eight clones, designated 1.23 and 1.28, had a low ouabain rescue coefficient (index of cooperation 0.35 and 0.33 respectively) (table 3.4). Subsequent ouabain rescue assays performed upon 1.23 have confirmed this (see table 3.11).

Clone 1.23 was further characterised for metabolic cooperation by uridine nucleotide transfer. The proportion of positive contacts as measured by uridine nucleotide transfer (section 2.72) was somewhat lower than that for B2B2TG5α (significant at the 5% level, see tables 3.11 and 3.13). Therefore I decided to pursue the selection further using 1.23 as starting material.

3.3 ISOLATION OF 1P9 FROM 1.23

5 x 10^7 1.23 cells were seeded onto four x 175cm² tissue culture flasks in medium containing 0.5μg/ml ICR-191. After 24 hours the mutagen was removed and the cultures were allowed to recover for 48 hours. 4 x 10^7 cells were recovered, mixed with 1.3 x 10^8 PC13.5 cells, and seeded into ten 175cm² tissue culture flasks. Selective conditions were applied on the following day, and

TABLE 3.4

Ouabain rescue indices of 1.23 and sibs

Cell Line	Ouabain	no. of colonies	Mean	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	734,713,713	720	0.49	1
	+	351,370,332	351		
1.21	-	468,418,498	461	0.46	0.95
	+	221,207,214	214		
1.22	-	350,358,358	355	0.22	0.45
	+	71,81,82	78		
1.23	-	518,474,560	517	0.15	0.31
	+	81,70,83	78		
1.24	-	278,311,265	285	0.49	1
	+	151,133,140	141		
1.25	-	859,892,897	877	0.46	0.94
	+	407,388,421	405		
1.26	-	587,604,681	624	0.49	1
	+	301,297,316	305		
1.27	-	506,427,508	480	0.43	0.88
	+	204,223,198	208		
1.28	-	248,262,295	268	0.17	0.35
	+	58,44,33	45		

maintained for 7 days. Cultures were maintained in unmodified medium for a further 10 days, by which time a number of large colonies were visible. In view of the fact that several colonies were present per flask, so that clonal populations in individual colonies could not be guaranteed, the colonies were trypsinised and pooled, and then expanded and cloned.

Twenty-two clones were obtained, designated 1P1-1P22 (1P15 was lost due to contamination). Two of these clones, 1P8 and 1P9, showed very low metabolic cooperation when assayed by ouabain rescue (index of cooperation 0.11 and 0.10 respectively) (table 3.5). 1P9 was characterised further and showed a significant reduction in intercellular nucleotide transfer (mean percent positive contacts = 0.7944, significantly different from B2B2TG5α at 1% level, see table 3.13).

3.4 ISOLATION OF 2P2[1]

In the hope of obtaining a cell line even more deficient in metabolic cooperation than 1P9, the remaining pooled survivors of the third round of selection were fed into another, highly stringent, kiss-of-death selection. 4 x 10^6 cells were mixed with 2.8 x 10^7 PC13.5 cells and seeded onto four x 175cm^2 tissue culture flasks. After overnight incubation they were fed with selective medium for 3 days then washed and cultures re-seeded with 10^7

TABLE 3.5

Ouabain rescue indices of 1P9 and sibs
(mean of 2 plates)

Cell Line	Ouabain	No. of colonies	Mean	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	797,699	748	0.39	1
	+	298,288	293		
1.23	-	961,1091	1026	0.106	0.27
	+	101,118	109.5		
1P1	-	530,609	569.5	0.149	0.38
	+	83,87	85		
1P2	-	570,540	555	0.149	0.38
	+	78,88	83		
1P3	-	504,522	513	0.148	0.38
	+	72,80	76		
1P4	-	602,635	618.5	0.119	0.31
	+	68,80	74		
1P5	-	473,483	478	0.113	0.29
	+	56,52	54		
1P6	-	749,782	765.5	0.099	0.25
	+	87,65	76		
1P7	-	310,320	315	0.106	0.27
	+	36,31	33.5		
1P8	-	510,580	545	0.043	0.11
	+	20,27	23.5		
1P9	-	645,694	669.5	0.039	0.10
	+	19,34	26.5		
1P10	-	643,789	716	0.112	0.29
	+	78,83	80.5		
1P11	-	771,740	755.5	0.093	0.24
	+	62,79	70.5		
1P12	-	995	995	0.075	0.19
	+	68,81	74.5		
1P13	-	807,804	805.5	0.092	0.24
	+	65,84	74.5		
1P14	-	555,593	574	0.086	0.22
	+	54,44	49		
1P16	-	493,483	488	0.075	0.19
	+	34,39	36.5		
1P17	-	717,709	713	0.099	0.25
	+	67,74	70.5		
1P18	-	677,607	642	0.074	0.19
	+	46,49	47.5		
1P19	-	677,677	677	0.11	0.28
	+	61,83	72		
1P20	-	660,578	619	0.06	0.15
	+	39,35	37		
1P21	-	558,513	535.5	0.095	0.24
	+	53,49	51		
1P22	-	473,475	474	0.088	0.23
	+	44,35	39.5		

PC13.5 cells per flask. Selective conditions were re-imposed on the following day, and maintained for 6 days. Then the cultures were allowed twelve days in unmodified medium, by the end of which time several large colonies were visible per flask. Twenty-two colonies were picked off (designated 2P1-2P22) and expanded for ouabain rescue assay (table 3.6). Of these, colony 2P2 was very poorly rescued and was cloned. Of the four resultant clones, one clone, designated 2P2[1], had a very low index of cooperation (0.035, table 3.7) and was chosen for further characterisation.

Clone 2P2[1] scores significantly lower than B2B2TG5α in the uridine transfer assay (mean proportion of positive contacts = 0.7688, significantly different from B2B2TG5α at the 1% level) (see table 3.13).

3.5 SELECTION OF COOPERATION-COMPETENT REVERTANTS FROM 1P9 AND 2P2[1] BY HAT RESCUE

As described in section 1.18, the HAT rescue selection or "kiss-of-life" depends upon metabolic cooperation occurring between "donor" HPRT$^+$ cells and "recipient" HPRT$^-$ cells. Aminopterin binds irreversibly to folate reductase, blocking the pathway for the de novo synthesis of Inosine Mono-Phosphate (IMP). HPRT$^+$ cells in HAT medium are able to overcome this block because the HPRT enzyme is able to convert Hypoxanthine in the medium to

TABLE 3.6

Ouabain rescue indices of 2P2 and sibs

Cell Line	Ouabain	no. of colonies	Mean	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	745,723	734	0.64	1
	+	461,479	470		
2P1	-	543,583	563	0.22	0.35
	+	114,134	124		
2P2	-	482,468	475	0.09	0.14
	+	52,34	43		
2P3	-	807,835	821	0.19	0.29
	+	137,175	156		
2P4	-	679,673	676	0.21	0.33
	+	141,143	142		
2P5	-	393,413	403	0.15	0.24
	+	71,49	60		
2P6	-	681,703	692	0.26	0.4
	+	186,174	180		
2P7	-	311,297	304	0.2	0.31
	+	66,56	61		
2P8	-	501,523	512	0.17	0.27
	+	75,99	87		
2P9	-	494,480	487	0.19	0.29
	+	88,98	93		
2P10	-	613,629	621	0.22	0.35
	+	144,130	137		
2P11	-	387,409	398	0.18	0.28
	+	67,77	72		
2P12	-	404,422	413	0.25	0.39
	+	93,113	103		
2P13	-	810,814	812	0.2	0.32
	+	174,150	162		
2P13	-	635,617	626	0.21	0.33
	+	141,121	131		
2P15	-	741,721	731	0.16	0.25
	+	103,131	117		
2P16	-	507,523	515	0.12	0.19
	+	66,58	62		
2P17	-	482,460	471	0.19	0.3
	+	94,84	89		
2P18	-	359,373	366	0.14	0.22
	+	48,54	51		
2P19	-	721,717	719	0.2	0.32
	+	138,150	144		
2P20	-	262,283	272	0.17	0.27
	+	46,48	47		
2P21	-	410,434	422	0.15	0.24
	+	72,54	63		
2P22	-	515,507	511	0.23	0.36
	+	111,125	118		

TABLE 3.7

Ouabain rescue indices of 2P2[1] and sibs
(mean of 2 plates)

Cell Line	Ouabain	no. of colonies	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	231	0.51	1
	+	117		
2P2[1]	-	380	0.018	0.035
	+	7		
2P2[2]	-	447	0.051	0.1
	+	23		
2P2[3]	-	432.5	0.058	0.11
	+	25		
2P2[4]	-	322	0.054	0.11
	+	17.5		

IMP. HPRT$^-$ cells are not able to do this and are consequently killed. When co-cultured with HPRT$^+$ cells, communication-competent HPRT$^-$ cells can be "rescued" from HAT toxicity by gap-junction mediated intercellular transfer of IMP. This phenomenon has been utilised to select the cooperation-competent revertant line H2T12 from the cooperation deficient EC variant R5/3 (Hooper and Morgan, 1979), and to select the revertant PT2mh1 from the partial revertant PT2mo1 (T.A. Smith and M.L. Hooper, pers. comm.). For the selection of PT2mo1 from the metabolic cooperation deficient EC line PT2md1, a modification of the ouabain rescue assay was employed (Smith T.A., 1984) (section 1.18). I decided against this approach for two reasons. Firstly, I wanted my selective system to resemble as closely as possible the kiss-of-death selection in reverse, i.e. to depend upon the passage of nucleotides via gap junctions. This requirement is fulfilled by HAT rescue but not by ouabain rescue. I consider this to be an important point: one could inadvertently select ouabain resistant variants by the ouabain rescue selection. Such variants would perform well in the ouabain rescue assay, but not by uridine transfer, so considerable time and effort could be wasted upon characterising a ouabain resistant, poorly cooperating variant. Of course a similar situation could arise with HAT rescue whereby cooperation deficient HPRT$^+$ revertants could be selected. However, such lines would be rapidly eliminated from further consideration by the initial ouabain rescue screen. The other consideration

was stringency of selection. In a standard ouabain rescue assay of a cooperation deficient cell line, 10-20 colonies typically survive out of an initial inoculum of 1000 cells. When this is multiplied up to millions of cells, it is likely that a substantial proportion of survivors would still be cooperation deficient. Although this problem can be largely overcome by prolonged ouabain treatment (17 days; Smith, 1984), reconstruction experiments using the HAT selective system at high cell densities (6 x 10^4 cells/cm^2, table 3.8) suggested that a highly efficient selection could be performed with just three days of HAT treatment. As prolonged culture is known to have a detrimental effect upon ES cell differentiation capacity, and incidentally increases the probability of contamination, it is important to minimise selection times.

Using HAT rescue I have isolated lines restored in their ability to communicate from the metabolic cooperation deficient lines 1P9 and 2P2[1]. The selections were performed in a single step, as described in section 2.94, and prior to selection cells were mutagenised with 1.5µg/ml ICR-191. The concentration of Hypoxanthine in the medium was increased from the usual concentration of 10^{-4}M to 10^{-2}M, as per the method described by Slack *et al* (1978). This facilitates the survival of cooperating $HPRT^-$ cells. After selection in HAT, cultures were maintained in unmodified medium until colonies were large enough to be picked off.

TABLE 3.8

Optimisation of HAT rescue selection; colony formation assay

(mean of 3 determinations on 60mm plates)

No. of 1P9 cells/plate	No. of B2B2TG5α cells/plate	HAT	No. of colonies	Survival frequency(%)
0	1000	-	520.6	52
0	1000	+	180	18
1000	0	-	481	48
1000	0	+	0	0
2.2×10^5	0	+	0	0
4.2×10^5	0	+	0	0
8.4×10^5	0	+	0	0
1.7×10^6	0	+	0.6	4×10^{-7}
3.4×10^6	0	+	2	6×10^{-7}
2.2×10^5	1000	+	197	N/A
4.2×10^5	1000	+	165	
8.4×10^5	1000	+	187	
1.7×10^6	1000	+	203	
3.4×10^6	1000	+	179	

In the case of the selection upon 1P9, 15 colonies were picked (11 were lost due to contamination). Of the four remaining colonies one, designated 2H4, showed a high ouabain rescue coefficient (index of cooperation = 0.9, table 3.9) and further characterisation revealed a high incidence of uridine nucleotide transfer (mean proportion of positive contacts = 0.9597, significantly different from 1P9 at the 1% level, not significantly different from B2B2TG5α).

From the selection upon 2P2[1], 22 colonies (designated H1-H22) were picked and expanded for ouabain rescue assay. Colony H19 had the highest index of cooperation (mean index = 0.86, table 3.10) and was therefore chosen for further characterisation. The mean proportion of positive contacts by uridine transfer was 0.9787, significantly different from 2P2[1] at the 1% level, not significantly different from B2B2TG5α.

The collated results of all ouabain rescue and uridine transfer assays are presented in tables 3.11, 3.12 and 3.13.

TABLE 3.9

Ouabain rescue indices of 2H4 and sibs

(mean of 2 plates)

Cell Line	Ouabain	no. of colonies	Mean	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	140,126	133	0.47	1
	+	55,71	63		
1P9	-	423,364	393.5	0.12	0.25
	+	40,56	48		
2H3	-	119,114	116.5	0.09	0.2
	+	10,12	11		
2H4	-	390,342	366	0.42	0.9
	+	168,140	154		
2H9	-	228,227	227.5	0.34	0.25
	+	92,63	77.5		
2H11	-	222,222	222	0.12	0.25
	+	25,28	27		

TABLE 3.10

Ouabain rescue indices of H19 and sibs
(mean of 2 plates)

Two separate experiments

Cell Line	Ouabain	no. of colonies	Mean	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	431,402	416.5	0.5	1
	+	218,196	207		
H1	-	467,494	480.5	0.31	0.62
	+	138,160	149		
H2	-	399,393	396	0.21	0.42
	+	75,91	83		
H3	-	493,429	461	0.24	0.48
	+	128,94	111		
H4	-	109,105	107	0.04	0.08
	+	4,5	4.5		
H5	-	613,548	580.5	0.045	0.09
	+	29,24	26.5		
H7	-	551,595	573	0.23	0.46
	+	115,147	131		
H8	-	544,497	520.5	0.32	0.64
	+	162,175	168.5		
H12	-	140,144	142	0.11	0.22
	+	16,16	16		

Cell Line	Ouabain	no. of colonies	Mean	Ratio (+:-)	Index of Cooperation
B2B2TG5α	-	1324,1382	1353	0.71	1
	+	951,969	960		
2P2[1]	-	798,867	832.5	0.12	0.17
	+	90,111	100.5		
H6	-	1209,1185	1197	0.14	0.2
	+	168,179	173.5		
H13	-	476,486	481	0.26	0.37
	+	120,132	126		
H14	-	815,787	801	0.52	0.73
	+	433,393	413		
H15	-	544,574	559	0.52	0.73
	+	285,295	290		
H16	-	1431,1470	1450.5	0.1	0.14
	+	156,134	145		
H17	-	889,925	907	0.52	0.73
	+	479,462	470.5		
H19	-	1147,1095	1121	0.61	0.86
	+	670,690	680		
H21	-	813,793	803	0.35	0.49
	+	277,284	280.5		
H22	-	445,550	497.5	0.55	0.77
	+	275,273	274		

TABLE 3.11

Metabolic cooperation indices of B2B2TG5α and variants; means of all experiments (including those experiments upon which selection of lines for further characterisation was based).

	Ouabain rescue		Uridine transfer	
Cell line	n	Mean index	n	Proportion of positive contacts
B2B2TG5α	13	1.000	8	0.9744
1.23	6	0.282	3	0.9091
1P9	8	O.216	6	0.7944
2H4	2	0.79	3	0.9597
2P2[1]	5	0.147	4	0.7688
H19	2	1.03	2	0.9787

TABLE 3.12

Statistical analysis of ouabain rescue results

Significance tests	$F_{1,81}$
B2B2TG5α vs 1.23	88.90
B2B2TG5α vs 2P2[1]	77.14
B2B2TG5α vs 1P9	212.9
B2B2TG5α vs H19	0.1214
B2B2TG5α vs 2H4	3.483
1.23 vs 2P2[1]	13·65
1.23 vs 1P9	1·321
2P2[1] vs H19	14.10
1P9 vs 2H4	42.24

F value for p = 5%	4.00
F value for p = 1%	7.08

TABLE 3.13

Statistical analysis of uridine transfer results

Significance tests	$F_{1,16}$
B2B2TG5α vs 1.23	7.392
B2B2TG5α vs 2P2[1]	39.00
B2B2TG5α vs 1P9	44.20
B2B2TG5α vs H19	0.0473
B2B2TG5α vs 2H4	1.092
1.23 vs 2P2[1]	3.085
1.23 vs 1P9	2.520
2P2[1] vs H19	10.28
1P9 vs 2H4	23.49

F value for p = 5%	4.54
F value for p = 1%	8.68

3.6 THE GROWTH HABIT AND DIFFERENTIATION PROPERTIES OF CELL LINES SELECTED IN THIS STUDY

All cell lines chosen for study, and the parent line B2B2TG5α were assayed for differentiation capacity by embryoid body formation and embryoid body outgrowths by the criteria given in sections 2.62 and 2.63.

Embryoid bodies were mainly scored after 7, 14 and 24 days in suspension. The bulk of the data is for 7 day embryoid bodies (and some at 6 days). The differentiation properties and growth habit of individual cell lines are described below.

The growth habit of B2B2TG5α, 1.23, 1P9 and 2H4 was very similar under standard culture conditions. Prior to confluence all three formed tightly packed nests of regularly shaped ES cells. At confluence a uniform, largely homogeneous mono-layer was formed, with only occasional differentiated cell types being evident in standard BRLcm. If cells were maintained for greater than a day at confluence the medium very rapidly became acidic resulting in cell death and differentiation. Cultures at this stage were very difficult to trypsinise. A single-cell suspension was almost impossible to achieve and aggregates tended to form upon subculturing, subsequently detaching from the surface to form primitive embryoid body-like structures. Therefore in order to maintain a healthy, homogeneous population a strict subculturing

regime was adhered to. On occasion, cells from a healthy culture trypsinised poorly and settled down to form aggregates upon subculturing (this seemed to be particularly prevalent with 1P9). However, in this case the aggregates flattened out after a day or so and normal growth ensued. 2P2[1] and H19 were rather different. At low density they formed loosely packed colonies of irregularly shaped cells, becoming very tightly packed and compact at confluence. If left for too long they tended to detach *en masse*, the entire cell layer lifting off the surface. Although difficult to trypsinise into a single-cell suspension at any time, their tendency to form aggregates was low compared to that of B2B2TG5α , 1P9 and 2H4, and while the occasional differentiated cell type was seen, it was usual for mono-layers of 2P2[1] and H19 to be highly uniform.

The mec$^+$ parental line B2B2TG5α and the mec$^+$ revertant from 1P9, 2H4, differentiated well in embryoid bodies and embryoid body outgrowths. Embryoid bodies at 6 and 7 days showed a high degree of endoderm formation and cavitation, with both parietal and visceral endoderm being present as well as other differentiated cell types such as blood islands and columnar epithelium (table 3.14, figures 3.5, 3.6 and 3.7, and plate 3.1). If left in suspension beyond 7 days, the proportion of cavitated embryoid bodies fell (table 3.14, figure 3.8 and plates 3.2 and 3.3). This seemed to be a consequence of cavities filling with large deposits of basement

TABLE 3.14

Differentiation of embryoid bodies of variants (6, 7, 14 and 24 days in suspension) All figures are expressed as % of the total

A: undifferentiated aggregate
B: differentiation of endoderm and secretion of matrix
C: formation of cavity

Cell line	expt.	days in suspension	Percentage of embryoid bodies in classes A	B	C	Total no. examined
B2B2TG5α	4	6	7	42	51	43
	1	7	0	34	66	53
	2	7	0	74	26	114
	3	7	2	62	36	64
	5	7	0	48	54	102
	6	7	0	48	57	54
	2B	14	0	64	36	36
	6B	14	0	65	35	20
	10	14	6	35	59	17
	6C	24	0	91	9	70
1.23	9	7	78	21	1	189
	9	7	53	41	6	126
	9	7	83	15	2	229
1P9	7	6	73	27	0	100
	1	7	40	58	2	46
	3	7	0	100	2	121
	5	7	0	98	2	45
	6	7	0	100	0	91
	6B	14	6	85	9	55
	6C	24	0	86	14	81
	11	24	11	72	17	18
	12	24	8	70	22	36
2H4	4	6	2	70	28	47
	8	6	3	29	68	190
	8	6	2	48	50	139
	3	7	0	61	39	68
	5	7	2	86	12	66
	6	7	0	91	9	46
	2B	14	0	76	24	29
	6B	14	14	75	11	36
	2C	24	0	84	16	45
	6C	24	0	94	6	67
2P2[1]	4	6	100	0	0	29
	7	6	100	0	0	71
	1	7	100	0	0	46
	2B	14	59	41	0	34
	2C	24	54	36	0	13
	11	24	100	0	0	20
H19	7	6	100	0	0	107
	5	7	80	20	0	35
	2B	14	33	67	0	16
	2C	24	39	61	0	31

Figure 3.5

Differentiation of 6 and 7-day old embryoid bodies formed from B2B2TG5α and variants

Abscissa = cell lines

Ordinate = percentage of embryoid bodies in category A, B or C

Note; 6-day embryoid body results are plotted on the left hand side, 7-days on the right

Type A
Type B
Type C
100
90
80
70
60
50
40
30
20
10
0
alpha 2H4 H19
1pp 2pZ(1)
1pp 2pZ(1)
alpha 2H4 H19

Figure 3.6

The proportion of 6 and 7-day old type B embryoid bodies containing AFP-positive staining material

Abscissa = cell lines

Ordinate = percentage of type B embryoid bodies

Note; shaded areas are equivalent to the percentage of type B embryoid bodies as plotted in figure 3.5

hatched areas represent the proportion of AFP-positive staining type B embryoid bodies.

6-day embryoid bodies are plotted to the left, 7-day embryoid bodies to the right

endoderm
AFP +ve
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
alpha 2H4 H19
1p9 2p21(1)
alpha 2H4 H19
1p9 2p21(1)

Figure 3.7

The proportion of 6 and 7-day old type C embryoid bodies containing AFP-positive staining material

Abscissa = cell lines

Ordinate = percentage of type C embryoid bodies

Note; shaded areas are equivalent to the percentage of type C embryoid bodies as plotted in figure 3.5

hatched areas represent the proportion of AFP-positive staining type C embryoid bodies.

6-day embryoid bodies are plotted to the left, 7-day embryoid bodies to the right

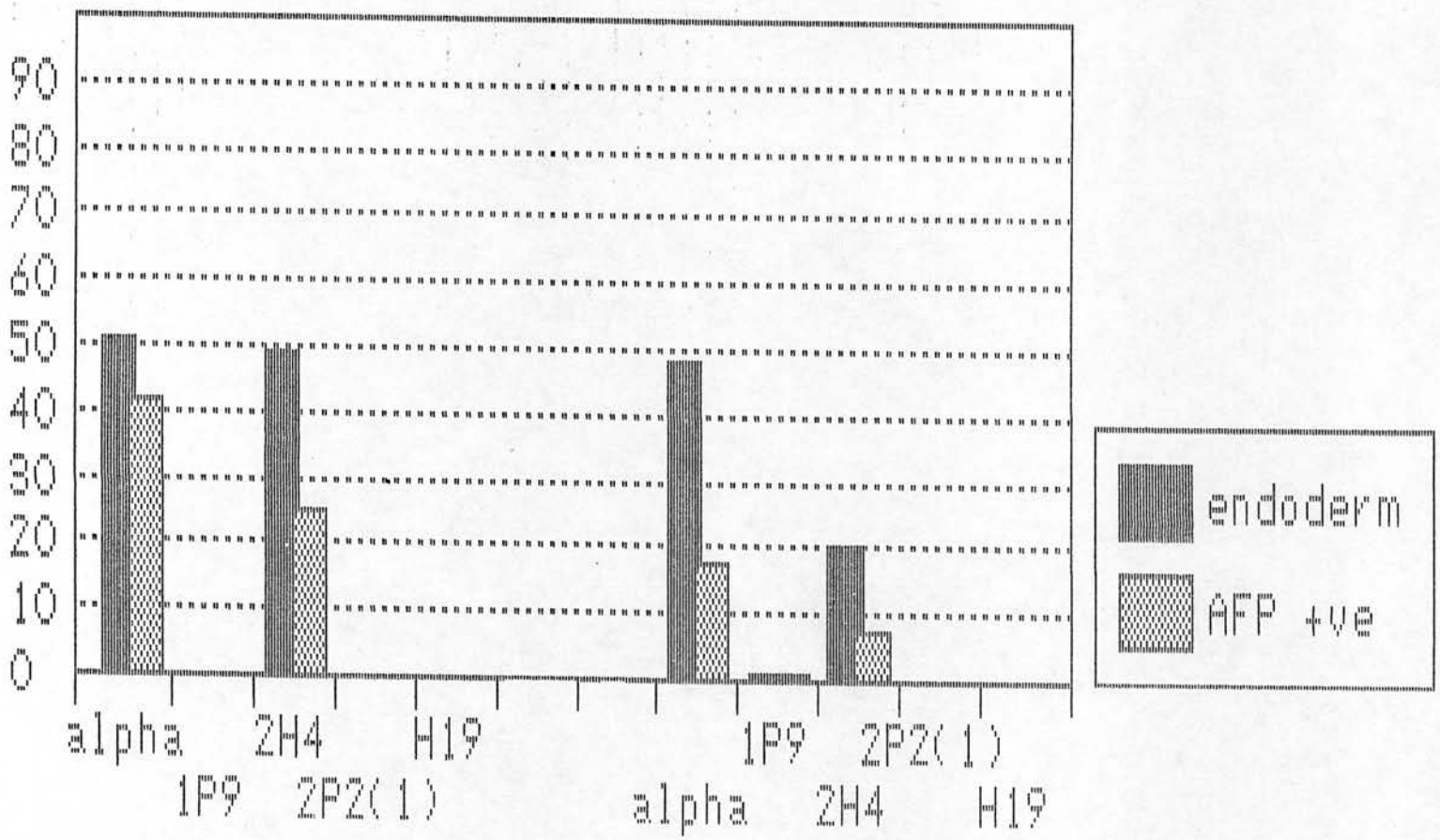

90
80
70
60
50
40
30
20
10
0
alpha
2H4
H19
1P9
2P2(1)
1P9
2P2(1)
alpha
2H4
H19
endoderm
AFF +VE

Figure 3.8

Differentiation of 14 and 24-day old embryoid bodies formed from B2B2TG5α and variants

Abscissa = cell lines

Ordinate = percentage of embryoid bodies in category A, B or C

Note; 14-day embryoid body results are plotted on the left hand side, 24-days on the right

Type A
Type B
Type C
100
90
80
70
60
50
40
30
20
10
0
alpha 2H4 H19
1P9 2P2(1)
alpha 2H4 H19
1P9 2P2(1)

PLATE 3.1

Photomicrographs of sections of embryoid bodies after 7 days in suspension.

A: B2B2TG5α (x19) (H & E)
B: B2B2TG5α (x77) (H & E)
C: 1P9 (x19) (AFP/PAS)
D: 1P9 (x77) (AFP/PAS)
E: 2H4 (x19) (AFP/PAS)
F: 2H4 (x77) (AFP/PAS)

Labels:

B = Blood islands
E = Endoderm
M = Extracellular matrix
PE = Parietal endoderm
VE = Visceral endoderm

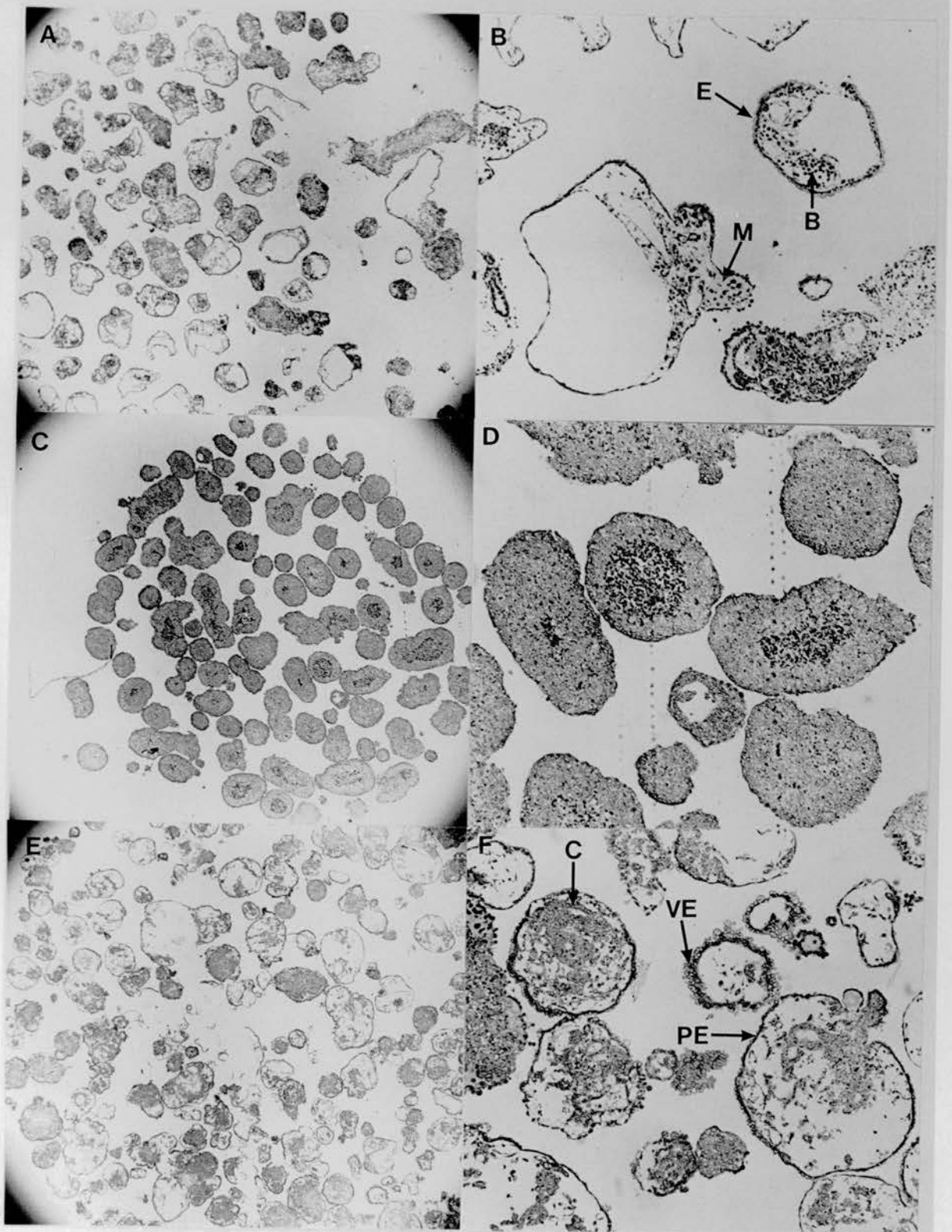

A
B
E
B
M
C
D
E
F
C
VE
PE

PLATE 3.2

Photomicrographs of sections of embryoid bodies after 24 days in suspension.

A: B2B2TG5α (x19) (H & E)
B: B2B2TG5α (x96) (H & E) Note large quantities of extracellular matrix material (arrowed)
C: 1P9 (x19) (AFP/PAS)
D: 1P9 (x77) (AFP/PAS)

Labels:

VE = Visceral endoderm
PE = Parietal endoderm

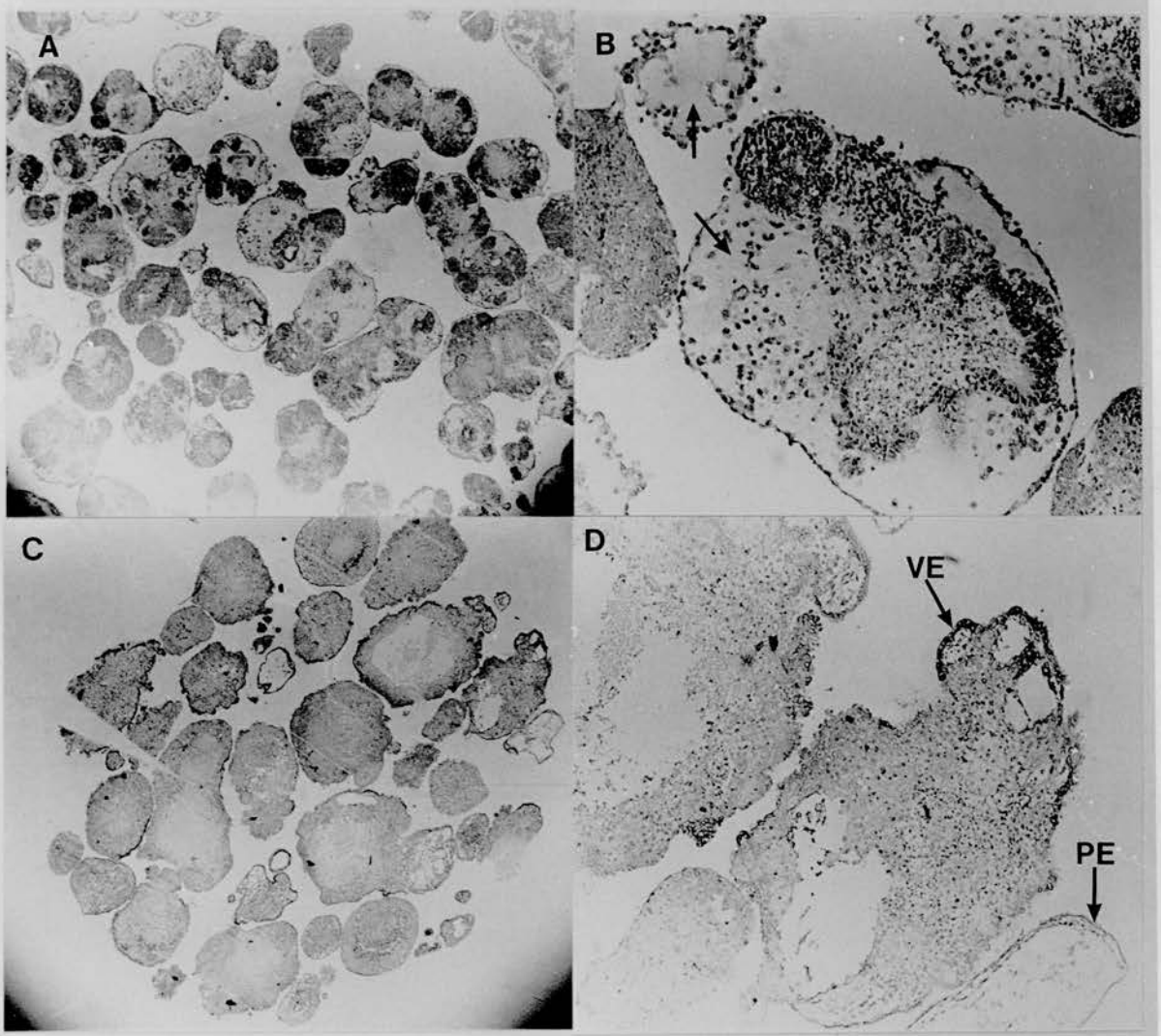
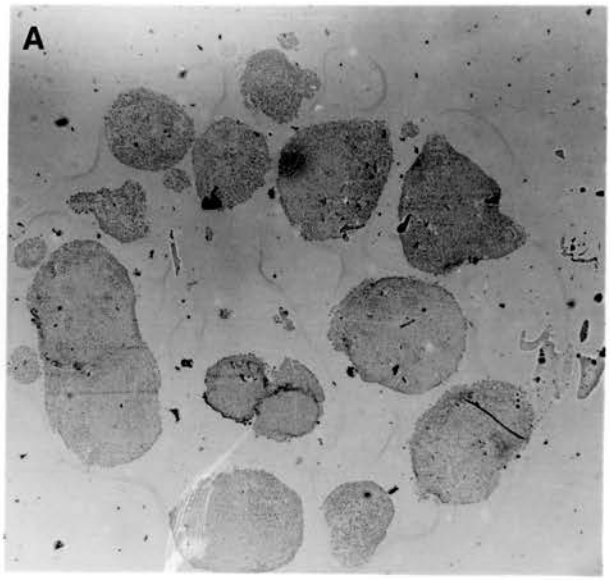
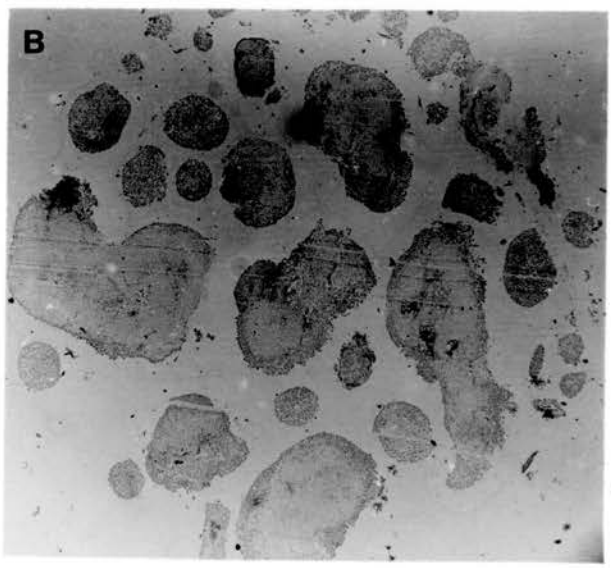

A
B
C
D
VE
PE

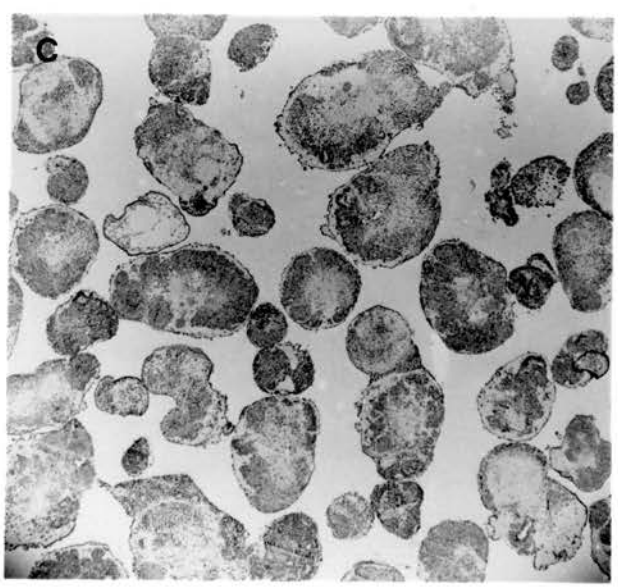
PLATE 3.3

Photomicrographs of haematoxylin and eosin stained sections of embryoid bodies after 24 days in suspension.

A: 2P2[1] (x19)
B: H19 (x19)
C: 2H4 (x19)


A


B


C

membrane, and possibly also with actively dividing cells. Unfortunately, only one determination with B2B2TG5α, and two with 2H4, were continued beyond 7 days so this observation should be treated with caution. Outgrowths formed from aggregates allowed to settle onto Thermanox coverslips (section 2.63) showed extensive differentiation, with trophectoderm-like cells, parietal endoderm, fibroblast-like cells and beating muscle being particularly prevalent (qualitative observation only; see plate 3.4 for examples).

The metabolic cooperation deficient variant 1P9 had an altered differentiation phenotype. At 6 and 7 days, embryoid bodies were poorly differentiated, with a reduced incidence of endoderm and no cavitation (table 3.14, figures 3.5, 3.6 and 3.7, and plate 3.1). If, however, 1P9 embryoid bodies were maintained in suspension for greater than 7 days, endoderm formation became extensive with thick profiles of basement membrane and occasional AFP-positive material, and cavitation became evident after 14 days of suspension culture, increasing in quantity up to 24 days (table 3.14, figures 3.8, 3.9 and 3.10, and plate 3.2). Cavitated 1P9 embryoid bodies at this stage have a rather unusual appearance. Some appear to consist entirely of a thin layer of strongly AFP-positive cells, with no other cell types being evident at all. Others superficially resemble wild type embryoid bodies at 7 days, in that they contain a number of differentiated cell types.

Figure 3.9

The proportion of 14 and 24-day old type B embryoid bodies containing AFP-positive staining material

Abscissa = cell lines

Ordinate = percentage of type B embryoid bodies

Note; shaded areas are equivalent to the percentage of type B embryoid bodies as plotted in figure 3.8

hatched areas represent the proportion of AFP-positive staining type B embryoid bodies.

14-day embryoid bodies are plotted to the left, 24-day embryoid bodies to the right

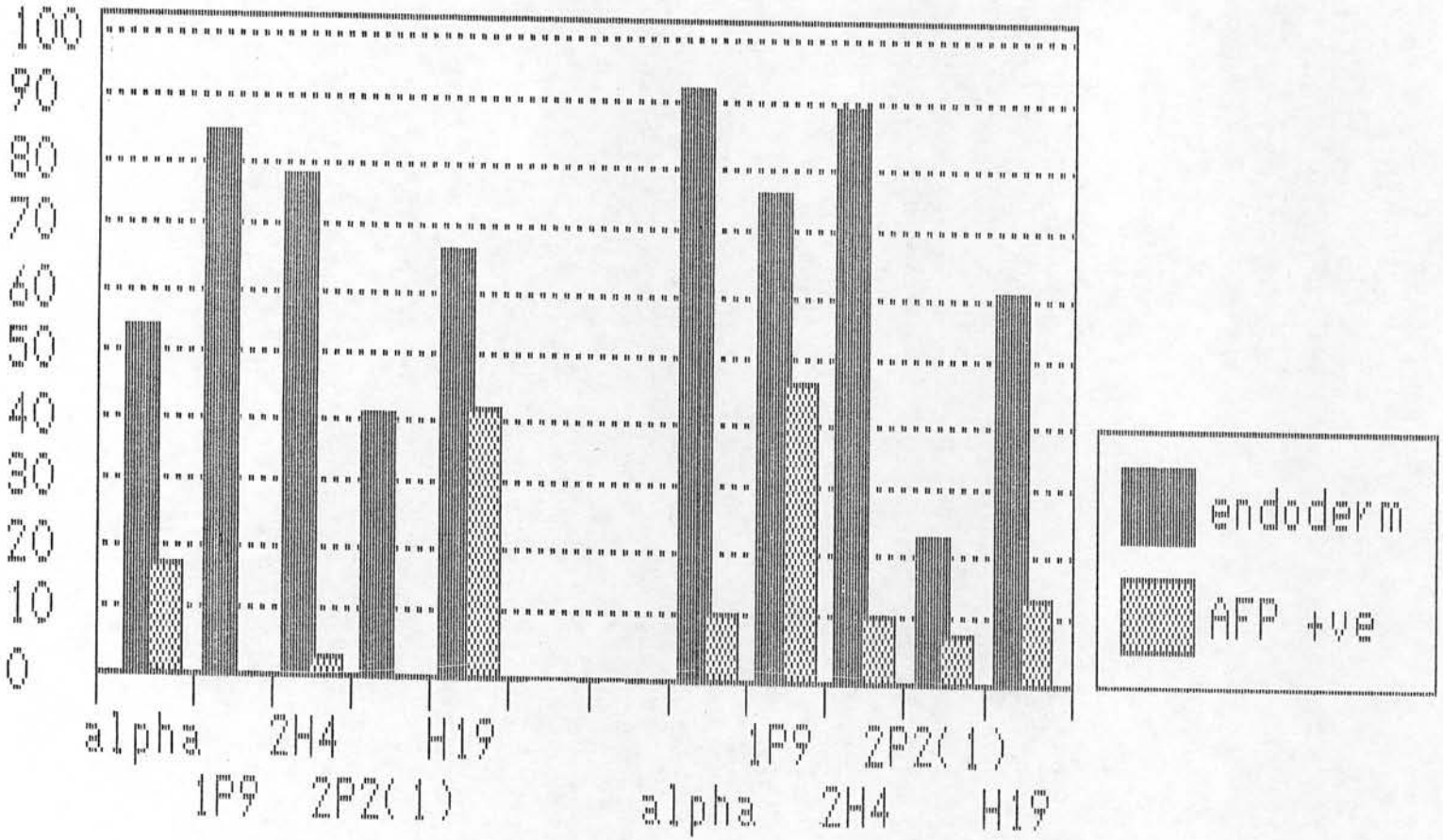

100
90
80
70
60
50
40
30
20
10
0
endoderm
AFP +ve
alpha
2H4
H19
1P9
2PZ(1)
1P9
2PZ(1)
alpha
2H4
H19

Figure 3.10

The proportion of 14 and 24-day old type C embryoid bodies containing AFP-positive staining material

Abscissa = cell lines

Ordinate = percentage of type C embryoid bodies

Note; shaded areas are equivalent to the percentage of type C embryoid bodies as plotted in figure 3.8

hatched areas represent the proportion of AFP-positive staining type C embryoid bodies.

14-day embryoid bodies are plotted to the left, 24-day embryoid bodies to the right

endoderm
dEP +ve
100
90
80
70
60
50
40
30
20
10
0
alpha 2H4 H19
1P9 2P2(1)
alpha 2H4 H19
1P9 2P2(1)

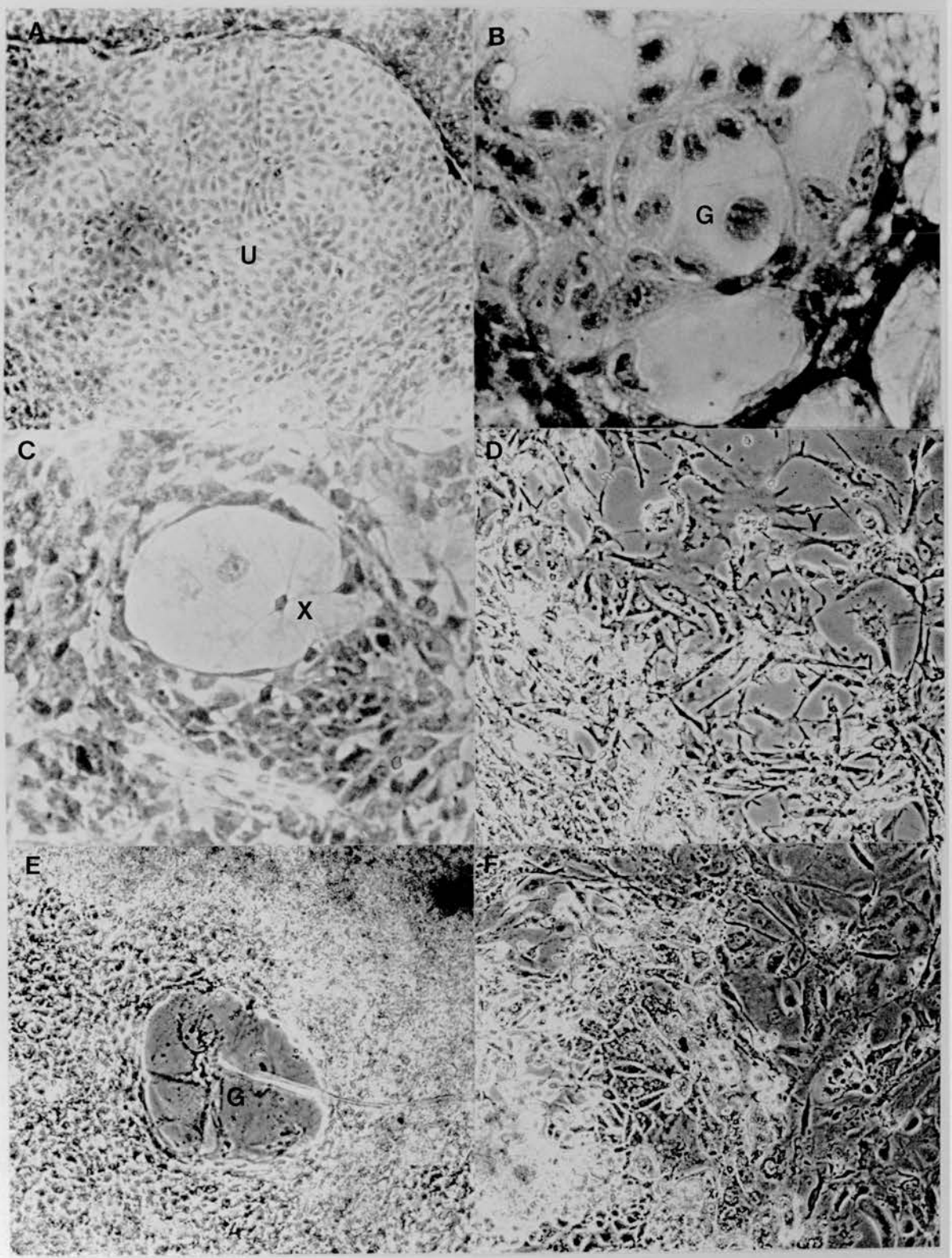
PLATE 3.4

Phase contrast photomicrographs of differentiated outgrowths from aggregates plated onto tissue culture dishes.

A: B2B2TG5α outgrowth (x120)
B: B2B2TG5α outgrowth (x360)
C: B2B2TG5α outgrowth (x306)
D: B2B2TG5α outgrowth (x120)
E: 2P2[1] outgrowth (x120)
F: 2P2[1] outgrowth (x96)

Labels:

U = Unidentified differentiated cell type
G = Cells resembling trophoblastic giant cells
X = Unidentified cell type showing multiple processes overlying a putative giant cell
Y = Large irregular cells with extensive cytoplasmic protuberances - one of the most common cell types to be found at the periphery of aggregate outgrowths of all cell lines tested


A
U
B
G
C
X
D
Y
E
G
F

However these embryoid bodies contain much larger quantities of basement membrane than is observed in cavitated wild type embryoid bodies at any stage between 6 and 24 days of suspension. In aggregate outgrowths the situation is very different, with differentiation appearing to mirror that of B2B2TG5α and 2H4 (plate 3.5). Based upon qualitative observations, there appears to be no difference between these cell lines (it should be noted that aggregate outgrowths were formed from embryoid bodies which had spent 6 days in suspension in all cases, and observations were made 14 days after this, so whether differentiation proceeds at a different rate initially remains open to question).

A high proportion of cavitated embryoid bodies from all three of the above lines do not appear to contain AFP-positive endoderm cells (table 3.15, figures 3.7 and 3.10). In view of the fact that cavitation is thought to be dependant upon the presence of visceral endoderm (section 1.8) this is a somewhat surprising observation. Possible reasons for this discrepancy will be discussed in section 3.8.

The intermediate rescue clone 1.23 appears to have a very similar phenotype to 1P9 with respect to embryoid body differentiation. However, only one experiment was performed and no direct comparison has been made. Also suspension times longer than 7 days have not been investigated. Embryoid body outgrowths have not been

TABLE 3.15

Immunocytochemical staining of embryoid bodies for alpha-foetoprotein

For this comparison, embryoid bodies are divided into two classes;

1: non-cavitated
2: cavitated

Within each class, the number of embryoid bodies containing (a) no endoderm; (b) endoderm of any type and (c) AFP-positive endoderm (a subset of category (b)) is expressed as a percentage of the total number of embryoid bodies in classes 1 and 2. Cavitation was not seen in the absence of endoderm. Percentages tabulated are mean values over all experiments.

		Percentage of embryoid bodies in each sub-group					
Cell line	days in suspension	Non-cavitated			Cavitated		Total no examined
		No endo.	Endo.	AFP+ve	Endo.	AFP+ve	
B2B2TG5	6	7	42	33	51	42	43
	7	0	52.2	15	47.8	17.2	431
	14	2	54.7	17.4	43.3	30.6	73
	24	0	92.5	10	7.5	3.5	70
1P9	6	73	27	15	0	0	100
	7	10	89	2.3	1	0.5	354
	14	6	85	0	9	0	55
	24	6.3	76	46.3	17.7	16	135
2H4	6	2.3	49	23.3	48.7	25.3	376
	7	0.7	79.3	10.7	20	7.2	180
	14	4.7	78.3	3	17	5.3	65
	24	0	89	10	11	3.5	112
2P2[1]	6	100	0	0	0	0	100
	7	100	0	0	0	0	46
	14	59	41	0	0	0	34
	24	77	23	7.5	0	0	33
H19	6	100	0	0	0	0	107
	7	80	20	0	0	0	35
	14	33	67	42	0	0	16
	24	39	61	13	0	0	31

Note: No endo = percentage of embryoid body sections containing no evidence of endoderm formation.

Endo = percentage of embryoid body sections containing endoderm cells (of any type).

AFP+ve = percentage of sections containing AFP-positive staining material.

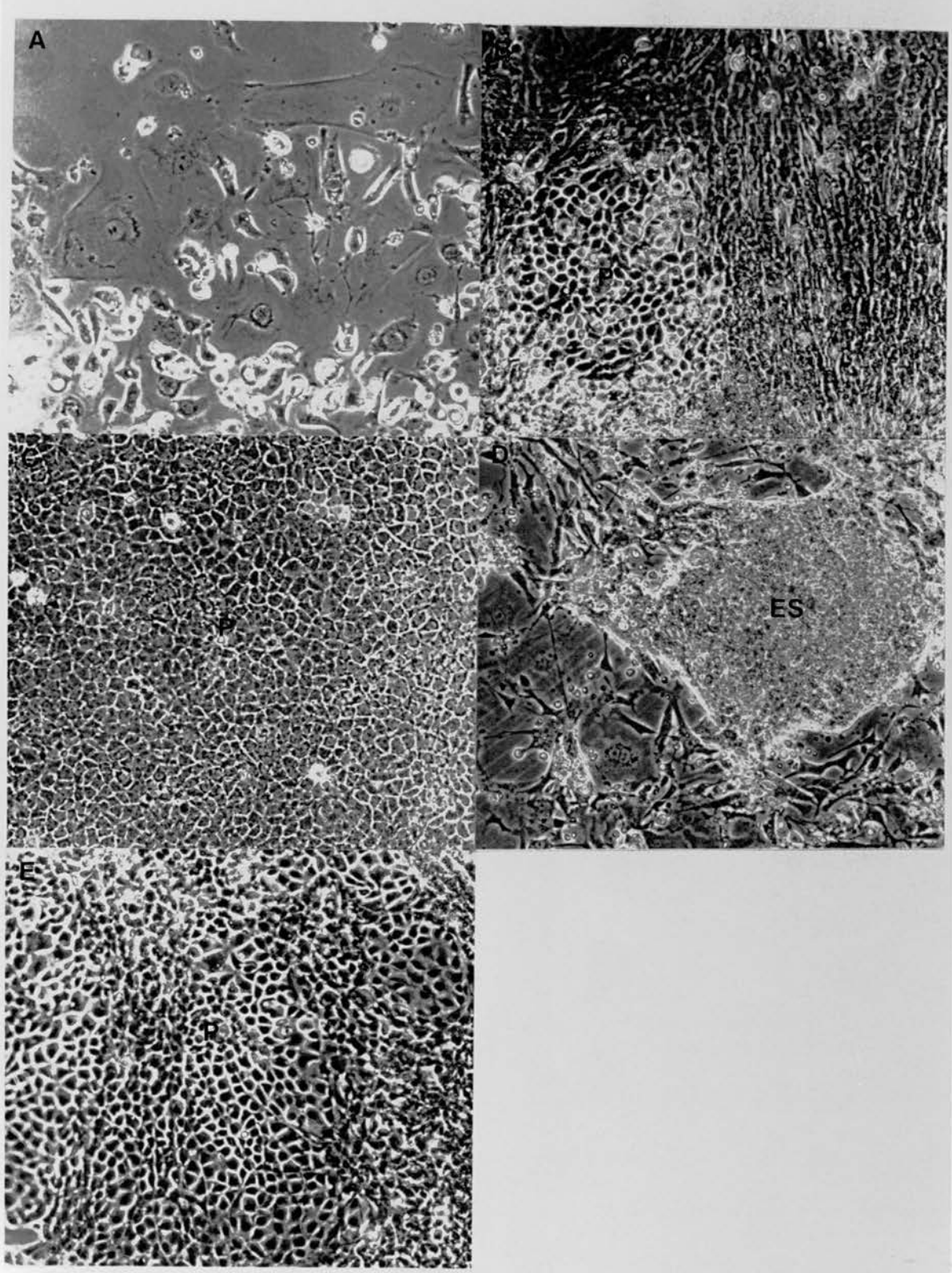
PLATE 3.5

Phase contrast photomicrographs of differentiated outgrowths from aggregates plated onto tissue culture dishes.

A: H19 outgrowth (x120)
B: H19 outgrowth (x120)
C: H19 outgrowth (x120)
D: 1P9 outgrowth (x120)
E: 1P9 outgrowth (x120)

Labels:

P = Parietal endoderm-like cells
ES = ES cells
R = Refractile cells


A
B
P
C
P
D
ES
E
P

formed from this line. In the light of results obtained from 1P9 this would seem to be an unnecessary exercise.

2P2[1] and H19 were very limited in their differentiation capacity. Neither cell line was capable of forming cavitated embryoid bodies, even after prolonged suspension culture, and aggregates remained largely homogeneous (table 3.14, figures 3.5 and 3.8, and plates 3.3 and 3.6). Occasionally thin regions of basement membrane accompanied by a small number of endoderm-like cells were visible and in some aggregates left for 14 and 24 days, a few cells staining positively for AFP were present. There was a slightly higher incidence of endoderm in H19 aggregates than in 2P2[1] aggregates. This observation is based upon one experiment only but is consistent throughout the 24 day duration of the experiment (determinations at 14 and 24 days) (table 3.15, figures 3.6 and 3.9).

If 2P2[1] or H19 aggregates are allowed to settle onto Thermanox coverslips, differentiated outgrowths become visible after a period of several days (plates 3.4 and 3.5). While these outgrowths are extensive in the spectrum of differentiated cell types present, they appear to take somewhat longer to establish themselves than outgrowths formed from B2B2TG5α, 1P9 or 2H4, and the proportion of ES-like cells present is considerably greater than with the latter (qualitative observations). This is probably a consequence of the relatively low

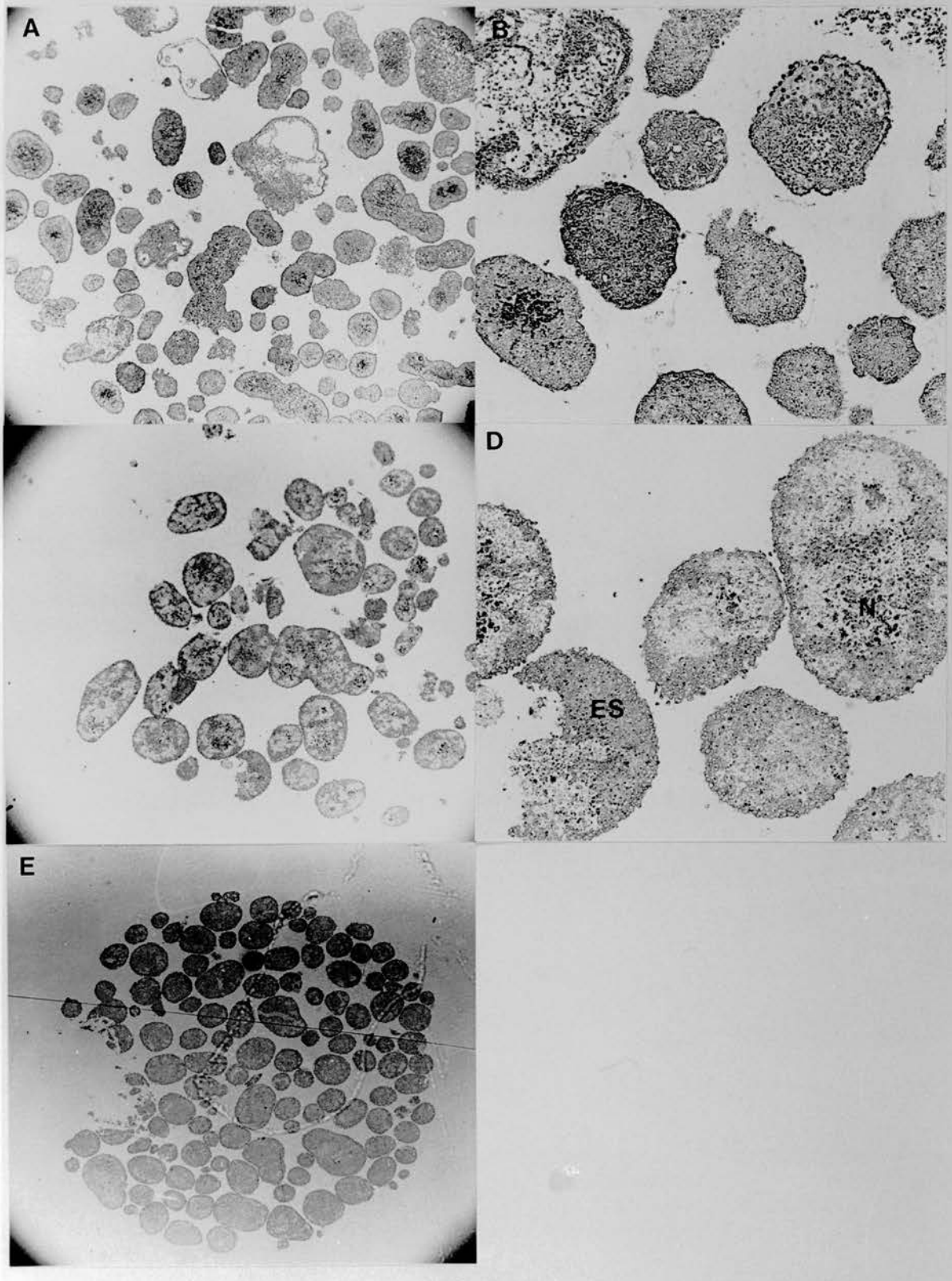
PLATE 3.6

Photomicrographs of haematoxylin and eosin stained sections of embryoid bodies after 7 days in suspension.

A: 1.23 (x19)
B: 1.23 (x77)
C: 2P2[1] (x19)
D: 2P2[1] (x77)
E: H19 (x19)

Labels:

ES = ES cells
N = Necrotic areas


A
B
D
N
ES
E

proportion of committed cells within 2P2[1] and H19 aggregates upon attachment. Again, both 2P2[1] and H19 behave in a similar manner.

The metabolic cooperation phenotypes of all variant cell lines, and their embryoid body differentiation phenotypes, are summarised in figure 3.11.

3.7 KARYOTYPES OF CELL LINES SELECTED IN THIS STUDY

To determine whether any of the variant phenotypes obtained in this study were due to gross chromosomal changes, metaphase spreads were prepared according to the method given in section 2.8, and 20 spreads from each cell line counted. All chromosomes were telocentric and no polyploidy was evident. All cell lines, including the B2B2TG5α parental, were aneuploid, the modal chromosome number being 43 for all but 2P2[1], which had a modal chromosome number of 44. However, almost as many spreads from 2P2[1] contained 43 chromosomes and this may well be a sampling error with a real mode of 43 for all lines. The original B2B2 stock was also karyotyped and found to contain a high proportion of cells having 43 chromosomes. As this original stock was at a high passage number upon receipt, presumably the aberrant karyotype has arisen prior to culture in our laboratory. Despite this, there seems to be nothing overtly wrong with the <u>in vitro</u> developmental capacity of these cells. Banding analysis

Figure 3.11

A summary of the metabolic cooperation and differentiation phenotypes of variants isolated in this study

Abscissa = ouabain rescue Index Of Cooperation

Ordinate = uridine nucleotide transfer proportion of positive contacts

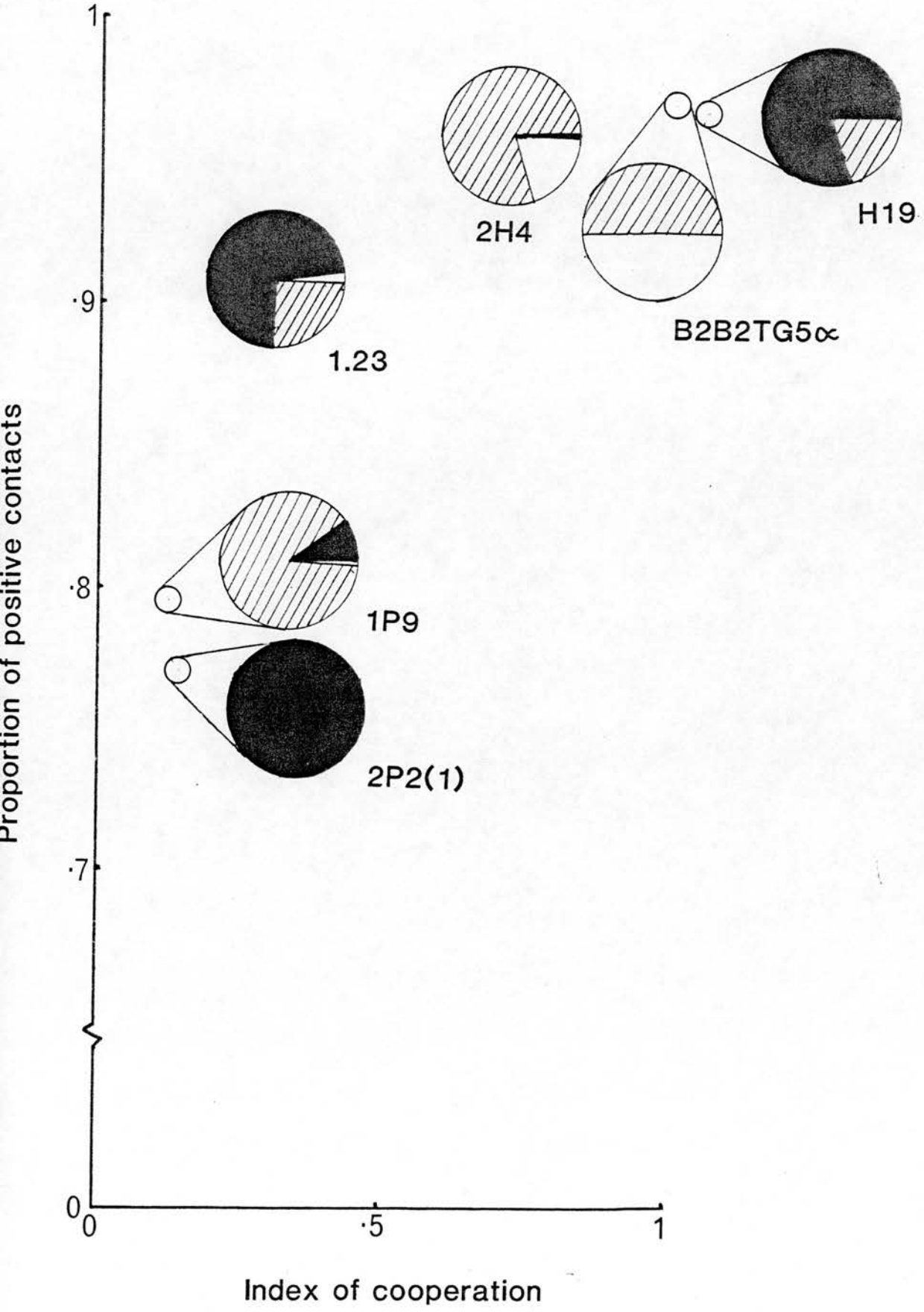
The centre of the pie indicates these parameters, except for those instances where the pies have been offset in order to avoid an overlap. Here, the small circles extrapolated from the pies indicate their real centre-points.

Pie charts represent mean values for embryoid body differentiation at 7 days

Shaded area = type A embryoid bodies

Hatched area = type B embryoid bodies

Open area = type C embryoid bodies


1
·9
·8
·7
0
0
·5
1
Proportion of positive contacts
Index of cooperation
2H4
H19
B2B2TG5∝
1.23
1P9
2P2(1)

was not carried out, although it would be of interest to find out which chromosomes have been duplicated. Chromosome counts are plotted in figure 3.12, and an example of a metaphase spread is presented in plate 3.7.

3.8 SUMMARY AND DISCUSSION

The thioguanine kiss-of-death and the HAT kiss-of-life are elegant selective techniques designed to isolate variant cell lines with altered capacity to perform gap junction mediated intercellular communication. This chapter describes a refinement of these techniques and their application to the ES cell line B2B2TG5, leading to the isolation in 3 steps and 4 steps respectively of the metabolic cooperation deficient lines 1P9 and 2P2[1], and to the subsequent isolation in 1 step of a cooperation competent revertant from each of these variants (2H4 and H19 respectively). For the purposes of this discussion I will deal with 2P2[1] and H19 first, since they are less interesting than 1P9 and 2H4.

The cooperation deficient variant 2P2[1] and its mec+ derivative H19 are both severely restricted in their ability to form endoderm and other differentiated cell types in aggregates in suspension culture, and are incapable of forming cavitated embryoid bodies, while being capable of forming a wide spectrum of differentiated tissues in aggregate outgrowths. Since

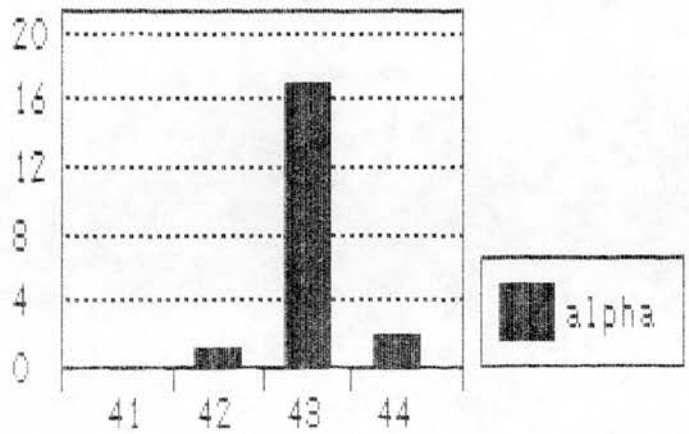
FIGURE 3.12

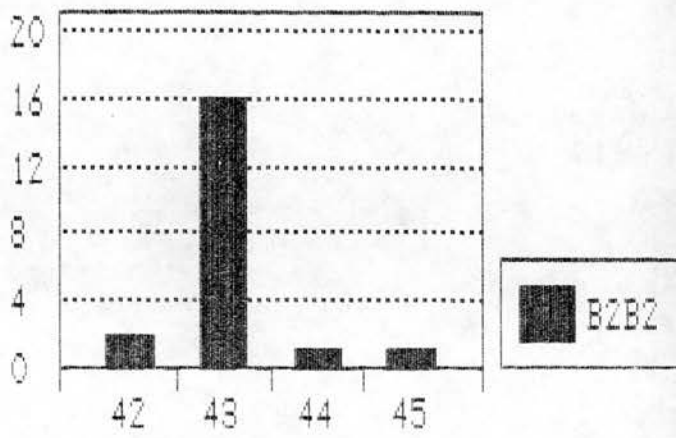
Karyotype distributions of variant cell lines

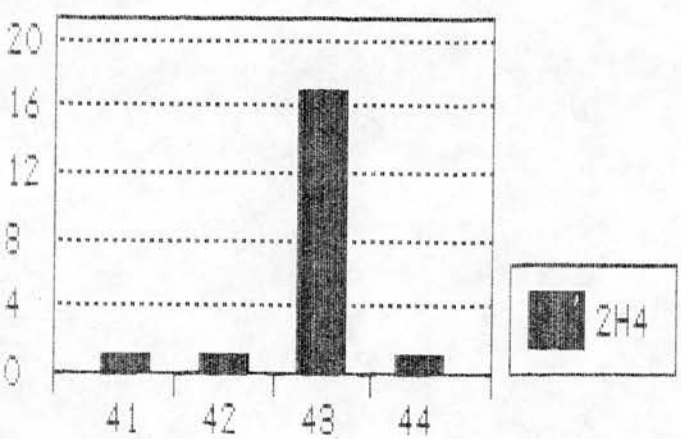
Abscissa = number of chromosomes

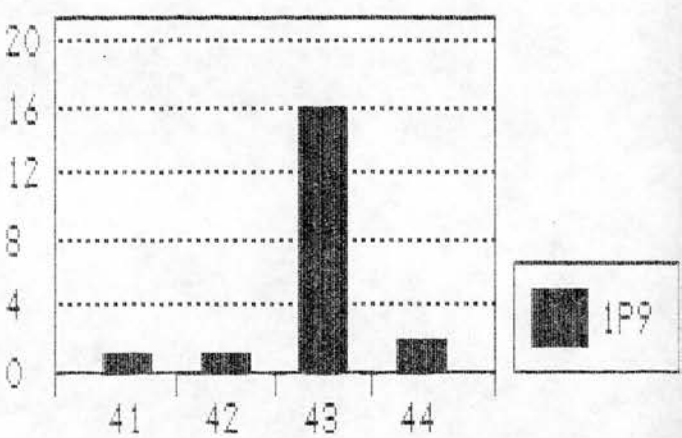
Ordinate = number of spreads

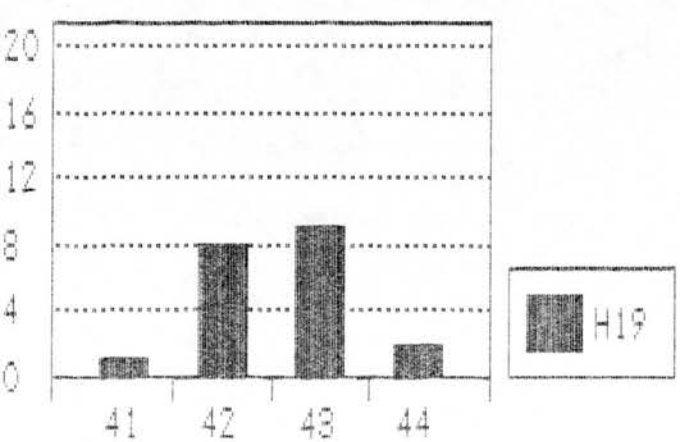
Note; the label "alpha" refers to B2B2TG5α


20
16
12
8
4
0
41
42
43
44
alpha


20
16
12
8
4
0
42
43
44
45
BZBZ


20
16
12
8
4
0
41
42
43
44
ZH4


20
16
12
8
4
0
41
42
43
44
1P9


20
16
12
8
4
0
41
42
43
44
H19

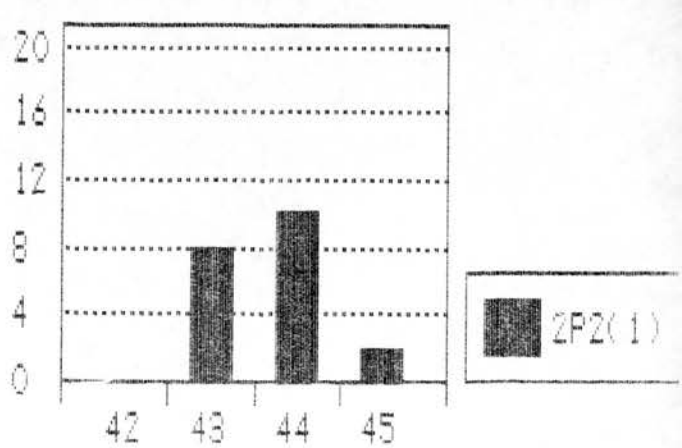
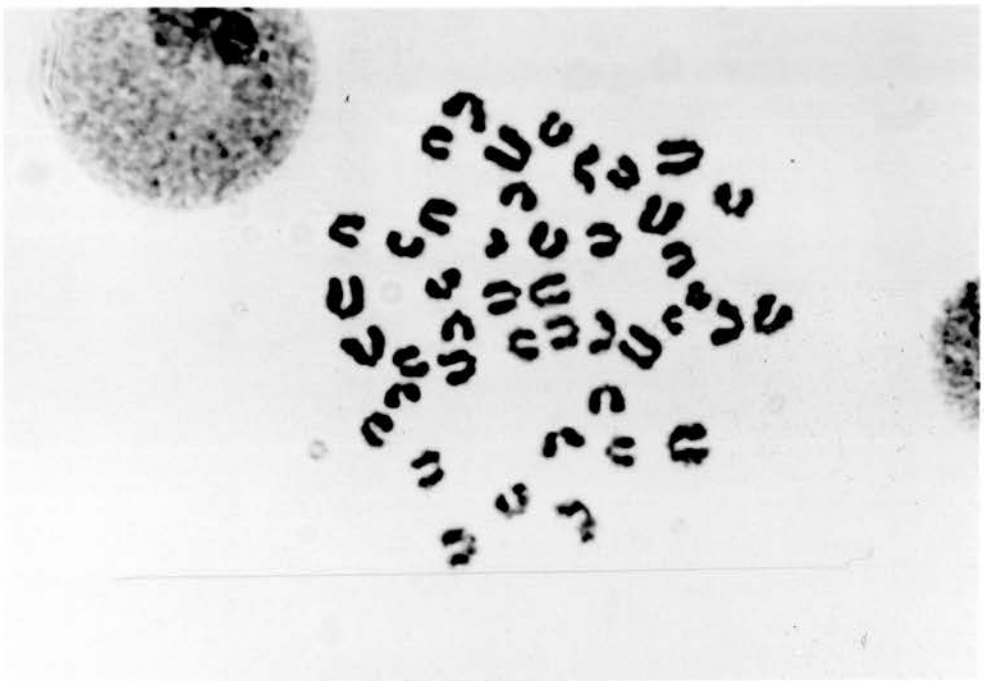

20
16
12
8
4
0
42
43
44
45
ZP2(1)

PLATE 3.7

B2B2TG5α metaphase spread containing 43 chromosomes (x1200).



cavitation in aggregates appears to be dependant upon the presence of an outer layer of endoderm (Martin *et al.*, 1977; Smith *et al.*, 1986), and where endoderm is observed in aggregates formed from these lines, it is present only in very small quantities (in no instance has a section from a 2P2[1] or H19 aggregate been completely surrounded by endoderm), the most attractive hypothesis to account for the embryoid body differentiation phenotype of 2P2[1] and H19 is that a secondary lesion has occurred in these lines reducing their capacity to form endoderm. The observation that no differentiation beyond limited endoderm formation takes place in aggregates of 2P2[1] and H19, whereas in aggregate outgrowths a wide spectrum of differentiated cell types emerge is a common phenomenon with nullipotent EC cells (section 1.8), and is consistent with the proposal of Martin and Evans (1975a) that cavitation is a necessary prerequisite for further differentiation by virtue of the provision of a two-dimensional surface which supplies positional information necessary for further cell determination to take place. In the case of aggregate outgrowths, this two-dimensional surface is provided by the substratum. Based upon qualitative observations it is noticeable that the aggregate outgrowths formed from 2P2[1] and H19 appear to contain a higher proportion of ES-like cells than outgrowths formed in parallel from B2B2TG5α, although there is no apparent difference with respect to the variety of differentiated cell types present. Also, differentiated outgrowths seem to take longer to emerge

from the 2P2[1] and H19 aggregates than from B2B2TG5α aggregates. Martin and Evans (1975b) have stated that the pattern of outgrowth differentiation is dependent upon the differentiation state of the embryoid bodies upon plating, and that the initial stage of outgrowth differentiation is the migration of a halo of endoderm from the aggregate onto the tissue culture substrate. Because observations of outgrowths in this study were in all cases made 14 days post-attachment, and quantitative analyses were not performed, my results yield no information relating to these observations. However, if endoderm outgrowth is a prerequisite for further differentiation, and if 2P2[1] and H19 are indeed restricted with respect to their rate of endoderm formation, then one would expect differentiated outgrowths from 2P2[1] and H19 aggregates to emerge more slowly than those from B2B2TG5α aggregates. It would be interesting, therefore, to clarify this situation by repeating the outgrowth experiments using the same methodology but making observations daily from day one of attachment.

The other cooperation deficient variant, 1P9, has a particularly interesting differentiation phenotype. Early observations of 6 and 7 day old suspension stage embryoid bodies indicated that the majority of 1P9 embryoid bodies contained endoderm at near wild-type levels, and cavitated embryoid bodies were present at very low frequency. Therefore the decision was taken to

maintain a culture of 1P9 embryoid bodies for an extended period to see if the proportion of cavitated embryoid bodies increased with time. This proved to be the case, with on average approximately 18% of embryoid bodies cavitated by 24 days in suspension. The appearance of these cavitated embryoid bodies was different to that of B2B2TG5α, however, with very broad profiles of basement membrane being present in the majority of cases, while in some instances cavitated embryoid bodies were composed of only a single-cell layer of endodermal cells staining very intensely for AFP. In B2B2TG5α embryoid bodies maintained in culture for 24 days there is a considerable increase in the quantity of basement membrane, to the extent that the majority of cavities appear to have been completely filled-in by it, and so this at least seems to be a normal consequence of extended culture periods for embryoid bodies of B2B2TG5-derived lines. The single-layered, AFP-positive type of embryoid body, however, appears to be unique to 1P9. The reason for this is not clear. This consideration aside, the important point here is that 1P9 is capable of forming cavitated, well-differentiated embryoid bodies but that to do so they require a much longer time in culture than the wild-type. Aggregate outgrowths from 1P9 are morphologically indistinguishable from those of B2B2TG5α after 14 days of culture post-attachment. Whether the outgrowths emerge at a different rate initially is still open to question, and although the 7 day old embryoid bodies formed from 1P9 possess a complete layer of

endoderm in the majority of cases, it is possible that the more advanced differentiation state of B2B2TG5α embryoid bodies at 7 days might lead to an increase in the initial rate of outgrowth differentiation. As suggested for 2P2[1] and H19, it will be necessary to perform a more detailed analysis of outgrowth formation to clarify this point.

In contrast to the revertant selected from 2P2[1], and indeed to revertant lines isolated from similar cooperation deficient variants by earlier workers, the cooperation competent revertant cell line 2H4, selected from 1P9, has also reverted to a near-normal differentiation phenotype. The possibility must be considered that 2H4 is a mec+ sibling which has survived unaltered throughout the kiss-of-death selection procedures to re-emerge from HAT selection. However, as the selection intermediate 1.23 is clonal and has a similar differentiation phenotype to that of 1P9, and 1P9 is also clonal, this is highly unlikely. It is concluded therefore that 2H4 is a true revertant from 1P9 and this being the case, that the altered differentiation phenotype of 1P9 is causally related to its metabolic cooperation deficiency. It is further concluded that in this system the major effect of the reduction in metabolic cooperation is to reduce the frequency with which cavitation takes place. The situation regarding outgrowth differentiation has not been analysed in sufficient detail to enable any conclusions to be drawn

at this stage, although it does appear that the spectrum of differentiation in monolayer may be unaffected.

It is evident from the results of the AFP/PAS stain upon embryoid bodies of B2B2TG5α, 1P9 and 2H4 that a high proportion of cavitated embryoid bodies show no evidence of AFP positive staining endoderm (visceral endoderm). This is a surprising observation, as it is thought that the presence of parietal or primary endoderm alone may not be sufficient to initiate cavitation (Martin et al., 1977; Grabel and Martin, 1983; Fisher, 1987), and so one would expect to observe a high proportion of cavitated embryoid bodies associated with visceral endoderm. There are several possible explanations for this, the most simple being that the cavitation process exhibits a requirement for endoderm which either visceral or parietal endoderm can fulfil, as suggested by Smith, Box and Hooper (1986). However, a more recent study by Fisher (1987) has revealed the presence of an as yet unidentified layer of endoderm-like cells in AFP-negative, parietal/primitive endoderm-negative cavitated embryoid bodies of the EC cell line PSA4TG12 and the ES cell line E14. This may be a precursor of mature visceral endoderm, and it is conceivable that it may also have been present, but not recognised, in those cavitated embryoid bodies which in previous studies were categorised as containing only parietal or primitive endoderm (Fisher, 1987). Unfortunately, in this study the third cell type was only scored if parietal/primitive

endoderm was absent and so this speculation requires confirmation. However, such a cell type was not obviously present in any of my sections. Also, it must be borne in mind that it is not possible to be confident of the absence of an endoderm type from an embryoid body on the basis of examination of a single section and it is likely that visceral endoderm was present in some embryoid bodies but out of the plane of section. It is impossible, therefore, to draw any concrete conclusions from the AFP/PAS staining results as they stand and in order to be able to do so, it will be necessary to perform a detailed analysis of serial sections of embryoid bodies - a task which was not undertaken in this study due to the constraints imposed by time.

The relationship between endoderm type and cavitation is an important point when one considers the apparent link between cavitation and metabolic cooperation, and the implications that this might have for the mechanism of cavity initiation in the early mouse embryo. By 4.5 days post-coitum, the developing blastocyst has implanted in the uterus wall, and at this stage the inner cell mass has developed into two distinct cell types; the primitive ectoderm which is destined to become the foetus, and the primary endoderm which creates a barrier between the primitive ectoderm and the blastocoel cavity. This primary endoderm then undergoes proliferation and further differentiation into parietal and visceral endoderm. The parietal endoderm is first to develop, and rapidly

migrates away from the ectoderm to form a continuous layer lining the inside of the blastocoelic cavity. Then the remaining primary endoderm differentiates into visceral endoderm, and it is this which lies in direct contact with the primitive ectoderm at the time of formation of the proamniotic cavity. It is tempting therefore to speculate that a signal is generated by the visceral endoderm which culminates in the initiation of the cavitation process, and, considering the embryoid body differentiation phenotypes of 1P9 and 2H4, that such a signal, or signals, may be passed from cell to cell via gap junctions. Once this initial step has taken place, the presence of the cavity may then stimulate further cell determination.

On the basis of the above hypotheses, and the results presented in this study, it becomes possible to evaluate various models for the initiation of the cavitation process. It seems reasonable to suppose that the differentiation of the endoderm layer is instigated by some mechanism which recognises the environmental difference between cells on the outside of the aggregate, and cells on the inside. Such inside/outside recognition has been shown to occur with blastomeres at the late 8-cell (compaction) stage (Ziomek and Johnson, 1980; Reeve and Ziomek, 1981; Reeve, 1981; Pratt *et al.*, 1982), and there is no reason to suppose that a similar mechanism cannot operate upon the outer layer of the embryoid body, or by implication the region of primitive

ectoderm adjacent to the blastocoelic cavity. In the following discussion I will explore the consequences of the assumption that the positional information necessary for cavitation is supplied by the endoderm layer and that morphogenetic substances involved with cavitation are able to pass from cell to cell via gap junctions. A further point which must be made at this stage is that metabolic cooperation defective cells do not consistently cooperate at a low level. Rather, the probability of junction formation once cells come into contact is reduced and it is conceivable therefore that a cooperation deficient cell experiences transient periods of high intercellular communication. Thus the rate of gap junction mediated transmission of any morphogenetic signal may vary in a random manner. As will become apparent, this is a crucial point when one attempts to reconcile the proposed models for cavitation with the ability of the cooperation defective variant 1P9 to form cavitated aggregates at low frequency.

The most simplistic models of cellular determination postulate a simple diffusion gradient of a morphogenetic signal passing from the outside of an aggregate to the inside, or vice versa (Saunders and Gasseling, 1968). Such a signal could act either by the inhibition of a differentiation pathway, or by its stimulation.

One may postulate that a morphogen capable of diffusing through gap junctions is uniformly distributed amongst,

and synthesised by, the cells of an undifferentiated aggregate and that a specialised function of the endoderm layer is to remove this substance either by metabolising it, or by transporting it into the medium. Provided that the net rate of morphogen removal by the endoderm becomes greater than or equal to its rate of synthesis within the aggregate, the formation of a layer of endoderm would lead to a concentration gradient, the morphogen being present at higher levels in the centre of the aggregate than at its periphery. Such a signal would necessarily be inhibitory, as a stimulatory signal under these conditions would be expected to initiate cavity formation in the absence of endoderm. Clearly, this is not consistent with the experimental data. If one therefore postulates the production of an inhibitory signal by the cells of the aggregate and its dissipation by the endoderm layer, one would expect cavitation to be initiated close to the edge of an aggregate. In the case of a cooperation defective cell aggregate cavitation would either fail to occur at all, or occur with low frequency at the periphery of the aggregate. Considering the probability model for metabolic cooperation discussed above, one would expect the frequency of cavitation to increase with time, and the experimental data show that this is indeed the case. Although on the basis of the data presented in this study it is not possible to entirely dismiss such a model, it must be called into question by qualitative observations of the pattern of cavity formation in 1P9 aggregates which indicate that

cavitation takes place at apparently random locations (although given that only a relatively small number of single sections were examined, and the position of cavities within those sections was not quantitatively analysed, this may not actually be so).

One can also construct a model whereby a cavitation-stimulating morphogen is produced by the endoderm layer and diffuses via gap junctions into the aggregate. Where the concentration of the activator reaches a certain value, cavitation is initiated. Again, one would predict that an aggregate composed of cooperation deficient cells would either fail to cavitate, or would cavitate at a low frequency with the frequency of cavitation increasing with time. As for the previous model, one would expect to see cavities predominantly at the periphery of aggregates, particularly in aggregates composed of cooperation-defective cells. The reservations expressed about the previous model therefore also apply to this model.

A much more elegant and satisfying model for cellular determination has been suggested by Meinhardt (1982). His proposal requires the production of two morphogenetic substances; the activator and the inhibitor. The activator is both autocatalytic and stimulates production of the inhibitor. The function of the inhibitor is to suppress the production of the activator. Therefore a spatially uniform steady state can arise across a field

of cells where production of activator and inhibitor precisely cancel one another out. If the diffusion rate of the inhibitor is higher than that of the activator, however, this steady state is unstable and if the level of activator is by chance elevated in a particular cell then it is possible for the inhibitor to fail to bring the level down again because it diffuses away into the surrounding cells more rapidly than the activator. The level of activator then becomes progressively higher until some limiting factor comes into play, for instance, the loss of activator by diffusion becomes equal to the net rate of production. In this way it is possible for a stable activator and inhibitor profile to be ultimately established from an initially homogeneous field of cells. A further consequence of this model is that the diffusion of the inhibitor will suppress activator production by neighbouring cells, the range of inhibition being dependant upon the rate of diffusion of the inhibitor and the rate at which it is broken down. Thus it is possible for a regular pattern to develop, with activation centres spaced apart from one another at very precise intervals. Meinhardt (1986) has shown that this model can be adapted to fit the process of segmentation in Drosophila. It is possible to apply this model to the cavitation process within embryoid bodies and within the early embryo. Let it be assumed that in this system the inhibitor diffuses via gap junctions and the activator diffuses by some other means, and that diffusion of inhibitor is more rapid than diffusion of activator. Let it also be

assumed that the putative cavitation activator is initially absent from the undifferentiated aggregate, and that its production is initiated by the endoderm layer, either by direct production of the activator itself or by production of some other diffusible activity which then stimulates the core cells to make activator. In both cases, activator production would rapidly become established throughout the entire aggregate, stimulating production of itself and of its inhibitor. At some stage the mechanism discussed above would be set into motion, and would result in a peak of activator at some point within the embryoid body (or primitive ectoderm). This would then develop into the cavity. If the diffusion rate of the inhibitor was sufficiently high, it would be possible for the existence of a single activation centre to suppress any further cavity formation within the aggregate. The formation of multiple cavities in a proportion of embryoid bodies, but not in the primitive ectoderm, may be due to factors related to differences in environment between naked ES cell aggregates in suspension culture and primitive ectoderm in the blastocyst. For example, the entire embryoid body is surrounded by endoderm whereas endoderm only comes into contact with approximately half of the surface of the primitive ectoderm in the early post-implantation embryo. It is conceivable that in embryoid bodies the activator substance could be produced simultaneously at a number of sites around the aggregate, and depending upon the kinetics of activator/inhibitor production and dispersal,

cavity activation centres might on occasion be established at multiple sites before the general concentration of inhibitor became sufficiently high to prevent further cavitation.

Applying this model to a cooperation defective cell line, it is evident that the rate of diffusion of inhibitor from a potential activation centre would be reduced. The consequence of this would be that the levels of inhibitor in any given locality would be subject to less fluctuation, and peaks of activator would therefore be generally suppressed. However, it is conceivable that on rare occasions metabolic cooperation could transiently become efficient enough to allow a local dissipation of inhibitor such that a peak of activator could develop, provided that the autocatalytic production of activator was sufficiently rapid. If this was the case, then one would expect to observe cavity formation at a low frequency, with the frequency increasing with time. Thus it is possible to utilise this model both to explain the experimental data obtained in this study and to tentatively propose a mechanism whereby secondary cavity formation might be prevented *in vivo*. The asymmetrical nature of cavity formation in both cooperation competent and cooperation defective embryoid bodies may also be accounted for, since it is clear that activation centres can arise at any point within the field of cells once the production of activator/inhibitor has become established throughout the aggregate. A particularly satisfying

aspect of this model is that it allows complex developmental patterns to emerge from an initially homogeneous population of cells, the only heterogeneity being, in this case, the production of endoderm on the outside of the aggregate and the initial pulse of the activator (or of an activity which stimulates activator production) from that endoderm.

Having said that, it is clear that until these elusive activator and/or inhibitor substances are positively identified, models such as these must remain purely hypothetical. Therefore it is not possible at this stage to confidently put forward any one model in preference to the others, and so all three of these proposed models, among others, must at the present time be considered as potential candidates for the mechanism of cavity initiation.

CHAPTER 4

THE EFFECT OF MUTAGENIC AGENTS UPON THE DIFFERENTIATION CAPACITY OF ES CELLS

4.1 INTRODUCTION

In view of evidence that the base substitution mutagen MNNG has a deleterious epigenetic effect upon chick lens cell transdifferentiation (Patek and Clayton, 1985), and possibly also upon EC cell differentiation (Smith *et al.*, 1986), it seemed prudent to investigate the effects of mutagens upon ES cell differentiation. I decided that there would be little point in attempting an investigation of genetic effects, as deleterious secondary lesions will inevitably occur in a proportion of variant cell lines anyway, and one must always bear this in mind when analysing the differentiation phenotype of selected variants which have been subjected to mutagenesis. My protocol cannot, however, exclude genetic effects since it is conceivable that a significant proportion of the cell population (i.e. sufficient to affect the outcome of the differentiation assay) could have acquired genetic lesions deleterious to their differentiation capacity, although I would consider this to be unlikely. Of more wide-ranging importance would be deleterious epigenetic effects, as these could affect a high proportion of the cell population, and thereby render meaningless any subsequent analyses of differentiation capacity. Therefore I have subjected populations of ES cells to mutagenesis with three different mutagens; the frame-shift mutagen ICR-191 (Summers, 1973; Wims and Morrison, 1981), the deletion mutagen 1,2,7,8,Di-Epoxy-Octane (DEO) (Huang *et al.*,

1978), and MNNG, and assayed these populations for their ability to form cavitated embryoid bodies.

In parallel with this, to ensure that the mutagens were being applied at levels sufficient to increase the mutation rate substantially, a proportion of cells used for the initial experiment were fed into a selection for resistance to 10μg/ml 6-thioguanine ($HPRT^-$). As the cell lines are both male-derived and the HPRT gene is X-linked (residing upon the region of the X chromosome which is non-homologous with the Y), they are hemizygous for HPRT, so this provides a straight-forward and accurate means to assess the efficacy of mutagenic agents.

4.2 THE EFFECT OF MUTAGENESIS UPON EMBRYOID BODY FORMATION

Three cultures of 3.5×10^7 E14 cells seeded onto 100mm tissue culture plates at a density of 2×10^4 cells/cm^2 were mutagenised as per the method given in section 2.4 with ICR-191 (0.5μg/ml), DEO (0.05mM) and MNNG (0.01mM). These levels were calculated from the survival vs concentration curves (figures 4.1, 4.2 & 4.3) to give a survival frequency of approximately 20%, although in practice the survival counts may have been biased by slow growth of a proportion of surviving mutagen-treated cells, and so the real survival frequencies may be somewhat higher than indicated. Following mutagenesis

FIGURE 4.1

Survival vs concentration plot for ICR-191 upon E14 cells plated at 2 x 10^4 cells per cm^2.

Abscissa = concentration of ICR-191 (µg/ml)

Ordinate = Percentage of surviving cells relative to the control.

TCR-191 toxicity (E14)
100
80
60
40
20
0
0
0.5
1
1.5
2
B

FIGURE 4.2

Survival vs concentration plot for DEO upon E14 cells plated at 2 x 10^4 cells per cm^2.

Abscissa = concentration of DEO (mM)

Ordinate = Percentage of surviving cells relative to the control.

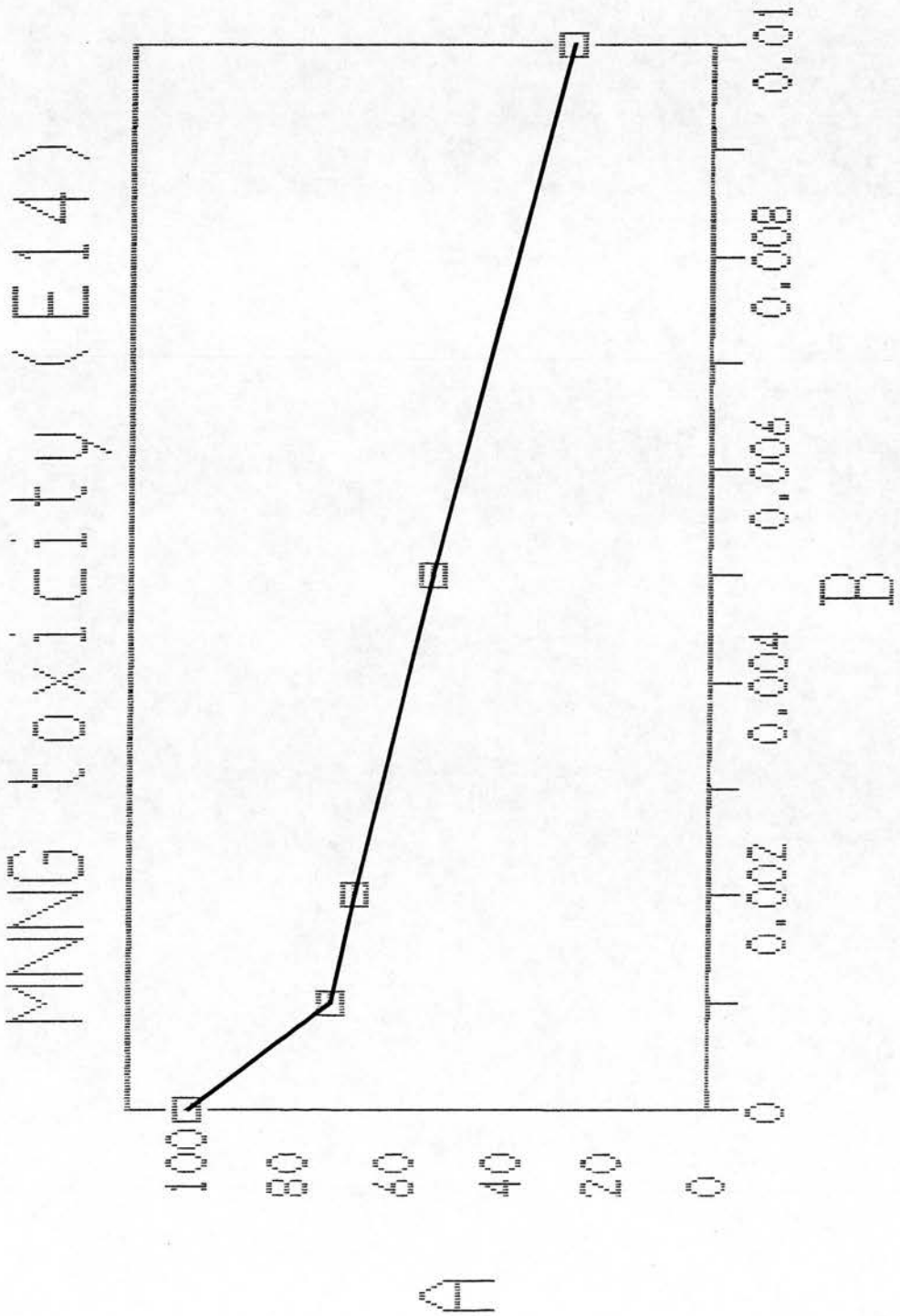
DEO toxicity (E14)
100
80
60
40
20
0
0
0.02
0.04
0.06
0.08
0.1
B

FIGURE 4.3

Survival vs concentration plot for MNNG upon E14 cells plated at 2 x 10^4 cells per cm^2.

Abscissa = concentration of MNNG (mM)

Ordinate = Percentage of surviving cells relative to the control.


MNG toxicity (E14)
T
100
80
60
40
20
0
0
0.002
0.004
0.006
0.008
0.01
B

the cultures were allowed to recover for 24 hours, and then they were trypsinised and re-seeded onto fresh plates to remove dead and dying cells, and 24 hours later re-trypsinised and survivors seeded onto 60mm tissue culture plates for aggregation (as per the method given in section 2.61). Embryoid bodies were prepared and scored in the usual manner. An untreated control culture was maintained in parallel and the experiments were carried out in duplicate.

Subsequent to this a similar experiment was set up using B2B2. Although identical in methodology, the cell density during mutagenesis was higher at 7 x 10^4 cells per cm^2, and mutagen concentrations were higher at 1.5μ g/ml ICR-191, 0.1mM DEO, and 0.02mM MNNG. The reason for this was that cells for the HAT selections had been mutagenised at this density and concentration of ICR-191 and the purpose of this experiment was to investigate the effect which mutagenesis might have had upon variant cell lines selected in this study. Kill curves indicated that the appropriate concentrations of DEO and MNNG were as given above. Survival vs concentration plots for the three mutagens are presented in figures 4.4, 4.5 and 4.6.

4.3 EMBRYOID BODY DIFFERENTIATION

Embryoid bodies formed from both E14 and B2B2 were extensively differentiated, with a high proportion

FIGURE 4.4

Survival vs concentration plot for ICR-191 upon B2B2 cells plated at 7×10^4 cells per cm^2.

Abscissa = concentration of ICR-191 (μg/ml)

Ordinate = Percentage of surviving cells relative to the control.

ICR-191 toxicity (B2B2)
100
80
60
40
20
0
T
0
0.5
1
1.5
2
2.5
B

FIGURE 4.5

Survival vs concentration plot for DEO upon B2B2 cells plated at 7 x 10^4 cells per cm^2.

Abscissa = concentration of DEO (mM)

Ordinate = Percentage of surviving cells relative to the control.

DEO toxicity (B2B2)
100
80
60
40
20
0
0
0.02
0.04
0.06
0.08
0.1
B

FIGURE 4.6

Survival vs concentration plot for MNNG upon B2B2 cells plated at 7 x 10^4 cells per cm^2.

Abscissa = concentration of MNNG (mM)

Ordinate = Percentage of surviving cells relative to the control.

MNG toxicity (B2B2)
100
80
60
40
20
0
T
0
0.01
0.02
0.03
0.04
0.05
B

possessing large, well-defined cavities and consisting almost entirely of differentiated cell types. No significant differences were apparent between control cultures and mutagen-treated cultures (tables 4.1 and 4.2, and figures 4.7 and 4.8).

4.4 CALCULATION OF MUTATION FREQUENCIES

Three cultures of E14 cells were mutagenised with ICR-191, DEO and MNNG and, along with a control culture set up in parallel, fed into a selection for resistance to 6-thioguanine as per the method given in section 2.42. Colonies of resistant cells were stained and counted and the induced mutation frequency for mutagenised cultures calculated in relation to the natural mutation frequency observed in control plates. The results are tabulated in table 4.3, and show an increase over the natural mutation frequency to $HPRT^-$ of 4, 4.5 and 7 times for DEO, MNNG and ICR-191 respectively.

4.5 DISCUSSION AND SUMMARY

This chapter describes an investigation of the effects of three mutagens; ICR-191, DEO and MNNG, upon the *in vitro* differentiation capacity of the ES cell lines E14 and B2B2.

TABLE 4.1

Embryoid body formation by mutagenised populations of E14 cells (determinations at 7 days).

Experiment 1

	aggregate type (absolute)			aggregate type (%)		
Mutagen	**A**	**B**	**C**	**A**	**B**	**C**
none	0	35	57	0	38	62
ICR-191	0	29	30	0	49	51
DEO	0	17	50	0	25	75
MNNG	0	62	60	0	51	49

Experiment 2

	aggregate type (absolute)			aggregate type (%)		
Mutagen	**A**	**B**	**C**	**A**	**B**	**C**
none	0	3	45	0	6	94
ICR-191	0	8	38	0	17	83
DEO	1	17	28	2	37	61
MNNG	0	5	24	0	17	83

TABLE 4.2

Embryoid body formation by mutagenised populations of B2B2 cells (determinations at 7 days).

Experiment 1

	aggregate type (absolute)			aggregate type (%)		
Mutagen	**A**	**B**	**C**	**A**	**B**	**C**
none	0	12	13	0	48	52
ICR-191	0	20	27	0	43	57
DEO	0	9	18	0	33	67
MNNG	0	33	11	0	75	25

Experiment 2

	aggregate type (absolute)			aggregate type (%)		
Mutagen	**A**	**B**	**C**	**A**	**B**	**C**
none	0	100	70	0	59	41
ICR-191	1	75	130	0	37	63
DEO	1	39	64	2	38	62
MNNG	0	19	45	0	30	70

TABLE 4.3

The increase in the frequency of mutation to HPRT- due to 0.5μg/ml ICR-191, 0.05 mM DEO and 0.01mM MNNG. Thioguanine resistant mutants selected from 10^7 B2B2 cells for each determination.

Mutagen	No. of TG resistant colonies	Increase in mutation frequency (relative to control)
none	2	1
ICR-191	14	7
DEO	8	4
MNNG	9	4.5

FIGURE 4.7

The differentiation state of 7-day embryoid bodies prepared from mutagenised populations of E14 cells.

Mean of two determinations. Bars represent the standard deviation about the mean.

Note: Type A, B and C refer to the categories of embryoid body defined in section 2.62.

Embryoid bodies - E14
Type A
Type B
Type C
100
80
60
40
20
0
%
control
IC0-191
DEO
MNNG
B

FIGURE 4.8

The differentiation state of 7-day embryoid bodies prepared from mutagenised populations of B2B2 cells.

Mean of two determinations. Bars represent the standard deviation about the mean.

Note: Type A, B and C refer to the categories of embryoid body defined in section 2.62.

Embryoid bodies - 8787
Type A
Type B
Type C
180
160
140
120
100
80
60
40
20
0
control
TCR-1A1
DEO
MNNG
B

There were two reasons for this investigation. Firstly, there is some evidence to suggest that MNNG mutagenesis may be linked to an alteration in the differentiation phenotype of some pluripotent and multipotent cell types, possibly via a mechanism involving changes in DNA methylation (Patek and Clayton, 1985; Lawley and Thatcher, 1970). In the case of EC cells this evidence is indirect, being based upon a statistical association between mutagenesis with MNNG and a reduction in differentiation capacity in subsequently isolated clones which had been subjected to kiss-of-death selection, and so a direct investigation into the effect of MNNG upon stem cell differentiation was considered to be of value. Secondly, it was necessary to evaluate the possible effects of ICR-191 mutagenesis upon my selected variants. DEO was included in the investigation since it was considered worthwhile to include a mutagenic agent with a different mode of action to that of MNNG and ICR-191.

The levels of mutagen employed here were all sufficient to elicit an increase in the frequency of mutation from $HPRT^+$ to $HPRT^-$ in the E14 test system. This was not tested with B2B2, although given that the levels of cell death in this case were similar to those of E14 under the conditions employed, it seems reasonable to assume that significant mutagenesis was taking place.

For the tests upon E14, cells were plated at low density so as to ensure log-phase growth at the time of

mutagenesis, based on the supposition that the effects of mutagenic agents were likely to be maximal at this stage. The cell density and mutagen concentrations employed for the experiments with B2B2 were chosen to mimic as closely as possible the most extreme conditions under which cells were mutagenised prior to my selections. It is notable that the toxicity of the mutagens to E14 cells was higher than to B2B2 cells under my test conditions. This may be due to a difference between the cell types with respect to their sensitivity to mutagenic agents, or to the higher cell density employed for B2B2 mutagenesis. I consider the latter explanation to be the more plausible, as it is possible that at the higher cell density the medium is conditioned to some extent by the ES cells, and that this contributes to a higher level of cell survival. At lower cell densities one would expect such non-specific feeder effects to be less marked.

Embryoid bodies formed from the mutagenised population appeared to be entirely normal, with little variation between control and test cultures within individual experiments, although there was considerable variation between experiments. This would appear at first sight to indicate that none of the mutagenic agents tested here exerts any effect upon the differentiation capacity of ES cells *in vitro*. However, it must be pointed out that the mutagenesis protocol here differs from that employed by Smith *et al* (1986), in that they mutagenised near-confluent cultures with levels of MNNG sufficient to

kill in excess of 90% of cells, and also that their analysis was upon clonal derivatives as opposed to entire cell populations. It is feasible that the levels of mutagen employed in this work were insufficient to elicit any detectable phenotypic changes with respect to embryoid body formation. Alternatively, it might be that cells at high density are more susceptible to MNNG mediated alterations in differentiation capacity than cells at lower density. It is difficult to see why this should be so.

In any case, under the conditions employed for ICR-191 mutagenesis prior to my selections, there are no detectable deleterious changes in the differentiation phenotype of B2B2 cells in mass culture. The isolation of a metabolic cooperation competent revertant cell line with restored differentiation ability (2H4) from the metabolic cooperation defective line 1P9, with restricted differentiation properties (chapter 3), lends weight to this contention.

CHAPTER 5

INHIBITION OF INTERCELLULAR COMMUNICATION BY 1-HEPTANOL AND 1- OCTANOL

5.1 INTRODUCTION

A number of previous reports have stated that the anaesthetic alcohols 1-heptanol and 1-octanol strongly and reversibly inhibit intercellular communication (Bernardini *et al.*, 1984; Ramon *et al.*, 1985). In this chapter I will describe the effects of heptanol and octanol upon intercellular communication in B2B2TG5α. Also, I will describe a preliminary analysis of the effect of heptanol upon PTmr0, a cell line selected from the EC line PSA4TG12 for resistance to inhibition of intercellular communication by 10^{-4}M retinoic acid (Smith *et al.*,1986).

5.2 1-HEPTANOL AND 1-OCTANOL STRONGLY AND REVERSIBLY INHIBIT INTERCELLULAR COMMUNICATION BETWEEN B2B2TG5α CELLS

Prior to carrying out any tests for intercellular communication, toxicity tests (section 2.5) were carried out around the concentrations claimed by Ramon *et al* (1985) to be effective in blocking metabolic cooperation, that is, 1mM octanol and 3mM heptanol. It was found that these levels were somewhat toxic to B2B2TG5α (approximately 40% mortality, figures 5.1 and 5.2).

Metabolic cooperation was initially assayed by ouabain rescue. The standard protocol was followed, octanol or

FIGURE 5.1

Survival vs concentration plot for 1-heptanol upon B2B2TG5α cells.

Abscissa = concentration of heptanol (mM)

Ordinate = Percentage of surviving colonies relative to the control.

See section 2.5

Heptanol toxicity
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
3.5
4
B

FIGURE 5.2

Survival vs concentration plot for 1-octanol upon B2B2TG5α cells.

Abscissa = concentration of octanol (mM)

Ordinate = Percentage of surviving colonies relative to the control.

See section 2.5

Octanol toxicity
100
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
B

heptanol being added at the same stage as ouabain and replaced daily for the duration of ouabain treatment. A range of concentrations for each alcohol was set up and control plates were supplemented with 0.1% (v/v) absolute ethanol. A small reduction in index of cooperation was observed at 3mM heptanol and 1mM octanol (index of cooperation = 0.625 and 0.7 respectively). Ouabain rescue indices are presented in table 5.1.

Uridine nucleotide transfer was then performed upon heptanol and octanol treated cultures. For the initial determination, the uridine nucleotide transfer was set up as normal, and recipient test plates were incubated overnight with either 3mM heptanol or 1mM octanol. On the following day, immediately prior to seeding with donor cells, test plates were re-fed with the appropriate test medium. Metabolic cooperation was strongly inhibited by both heptanol and octanol, the proportion of positive contacts being 0.188 and 0.499 respectively as compared to the control value of 0.983 (table 5.2). A second determination was then carried out to confirm this result and also to investigate the rapidity and reversibility of inhibition (insofar as the experimental system allowed). This time, plates were set up as before (with the addition of one set treated with 2mM heptanol) but four extra plates were set up for treatment with 3mM heptanol. Two of these plates were incubated overnight with heptanol then washed and replenished with unmodified medium upon seeding with donor cells. The other two

TABLE 5.1

Ouabain rescue indices of B2B2TG5α cells treated with heptanol and octanol (mean of 3 determinations). Initial inoculum 500 cells/plate.

Heptanol (mM)	Octanol (mM)	Ouabain	No. of colonies	Ratio (+:-)	I.O.C.
-	-	-	390	0.4	1
-	-	+	157		
0.1	-	-	399	0.41	1.025
0.1	-	+	164		
1	-	-	369	0.36	0.9
1	-	+	134		
3	-	-	238	0.25	0.625
3	-	+	59		
-	0.1	-	388	0.42	1.075
-	0.1	+	167		
-	0.5	-	389	0.33	0.825
-	0.5	+	128		
-	1	-	283	0.28	0.7
-	1	+	80		

I.O.C. = Index Of Cooperation

TABLE 5.2

Uridine nucleotide transfer upon heptanol and octanol treated cultures of B2B2TG5α cells.

Conc. of heptanol (mM)	Conc. of octanol (mM)	proportion of positive contacts	
		Experiment 1	Experiment 2
0	0	0.983	0.985
2	-		0.766
3	-	0.188	0.560
3(u/s)	-		0.398
3(r/s)	-		1.000
-	1	0.449	0.648

u/s = heptanol added upon seeding with donors
r/s = heptanol removed upon seeding with donors following 24 hour incubation

plates were left overnight in unmodified medium, and then fed with medium containing 3mM heptanol upon seeding with donor cells. Control cultures were supplemented with 0.1% (v/v) absolute ethanol. Metabolic cooperation was strongly inhibited in cultures incubated overnight with 1mM octanol or 3mM heptanol, and also in cultures to which 3mM heptanol had been added immediately prior to seeding (inhibition appeared to be slightly greater in the latter, but as only one determination was carried out this could have been an artifact). Metabolic cooperation was restored to control levels in the culture from which heptanol had been removed prior to seeding. Inhibition of metabolic cooperation was detectable at 2mM heptanol, but much reduced compared to levels observed at 3mM. These results are presented in table 5.2, and an example of a typical ^{3}H-uridine transfer preparation with and without 3mM heptanol is shown in plate 5.1.

The results of the uridine transfer assay demonstrate that 1-octanol and 1-heptanol both inhibit metabolic cooperation between B2B2TG5α cells, and this inhibition is rapidly established and reversible, at least in the case of heptanol. The anomalously high cell survival in the ouabain rescue assay is most probably due to rapid evaporation of heptanol and octanol from test plates.

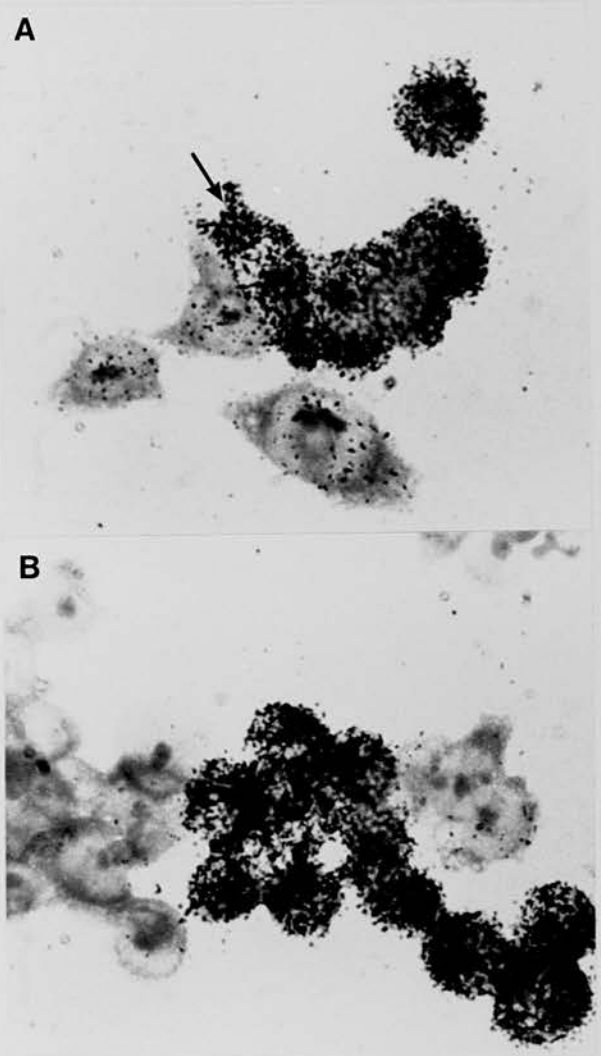
The primary objective of this exercise was to utilise inhibitors of intercellular communication to investigate further the relationship between metabolic cooperation

PLATE 5.1

Typical appearance of ^{3}H-uridine nucleotide transfer autoradiographs (x768).

A: B2B2TG5α
B: B2B2TG5α + 3mM 1-heptanol

A donor cell is arrowed.


A
B

and differentiation. However, in view of the prolonged periods of time required for both aggregation and embryoid body formation, and monolayer differentiation, the toxicity of octanol and heptanol proved to present an insurmountable obstacle. Therefore this line of investigation had to be abandoned. It did however suggest another avenue of investigation which proved to be more fruitful, namely, the further characterisation of the metabolic cooperation variant PTmr0. This work is discussed in the following section.

5.3 THE EFFECT OF HEPTANOL UPON INTERCELLULAR COMMUNICATION IN PTmr0

It has been known for some time that retinoic acid at a high concentration is a potent inhibitor of metabolic cooperation (Pitts *et al.*, 1981). The variant PTmr0 was selected for resistance to the inhibition of metabolic cooperation by 10^{-4}M retinoic acid. Knowing that octanol and heptanol also inhibit intercellular communication, it seemed pertinent to ask whether the mode of action of retinoic acid-induced inhibition is the same as, or distinct from, that of octanol and heptanol. PTmr0 presented an opportunity to answer that question.

Uridine nucleotide transfer assays were set up with PTmr0 and PSA4TG12. Individual test plates were incubated with 10^{-5}M or 10^{-4}M retinoic acid, or 1, 2 or 3mM heptanol for

thirty minutes prior to seeding with homotypic donor cells. Retinoic acid or heptanol treatment was maintained for the duration of co-culture. The standard uridine transfer protocol was then followed. In two separate experiments PTmr0 showed very little response to retinoic acid treatment, but a marked reduction in grain counts was observed in plates treated with 3mM heptanol (table 5.3, figures 5.4 and 5.5). In the first determination PSA4TG12 showed very little response to either retinoic acid or heptanol. In the second determination however, PSA4TG12 showed a marked drop in intercellular communication in response to both 10^{-4}M retinoic acid and 3mM heptanol (table 5.3, figures 5.3, 5.4 and 5.5). The reasons for this variation between experiments are not clear. Despite this anomaly though, the response of PTmr0 in both experiments was unambiguous, demonstrating inhibition of metabolic cooperation by 3mM heptanol but not by 10^{-4}M retinoic acid. The implications of this observation will be considered further in the next section.

5.4 DISCUSSION AND SUMMARY

In this chapter the effects of 1-heptanol and 1-octanol upon intercellular communication in B2B2TG5α, and of 1-heptanol upon intercellular communication in PSA4TG12 and PTmr0, are described.

TABLE 5.3

Uridine nucleotide transfer upon cultures of PSA4TG12 and PTmr0 treated with heptanol and retinoic acid.

Cell line	Conc. of heptanol (mM)	Conc. of retinoic acid (mM)	Proportion of positive contacts Expt. 1	Expt. 2
PSA4TG12	0	0	0.987	0.990
	0	0.01	0.987	0.941
	0	0.1	0.987	0.333
	1	0	0.974	0.990
	2	0	0.947	0.562
	3	0	0.941	0.422
PTmr0	0	0	0.987	0.990
	0	0.01	0.987	0.990
	0	0.1	0.987	0.852
	1	0	0.987	0.962
	2	0	0.702	0.990
	3	0	0.247	0.464

FIGURE 5.3

Uridine nucleotide transfer indices for PSA4TG12 and PTmr0 cells incubated for 24 hours with retinoic acid (experiment 2).

Abscissa = concentration of retinoic acid (mM)

Ordinate = proportion of positive contacts

PS4TG12/PTmPO + retinoic acid
PS4TG12
PTmPO
1
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
0.001
0.01
B

FIGURE 5.4

Uridine nucleotide transfer indices for PSA4TG12 and PTmr0 cells incubated for 24 hours with heptanol (experiment 1).

Abscissa = concentration of heptanol (mM)

Ordinate = proportion of positive contacts

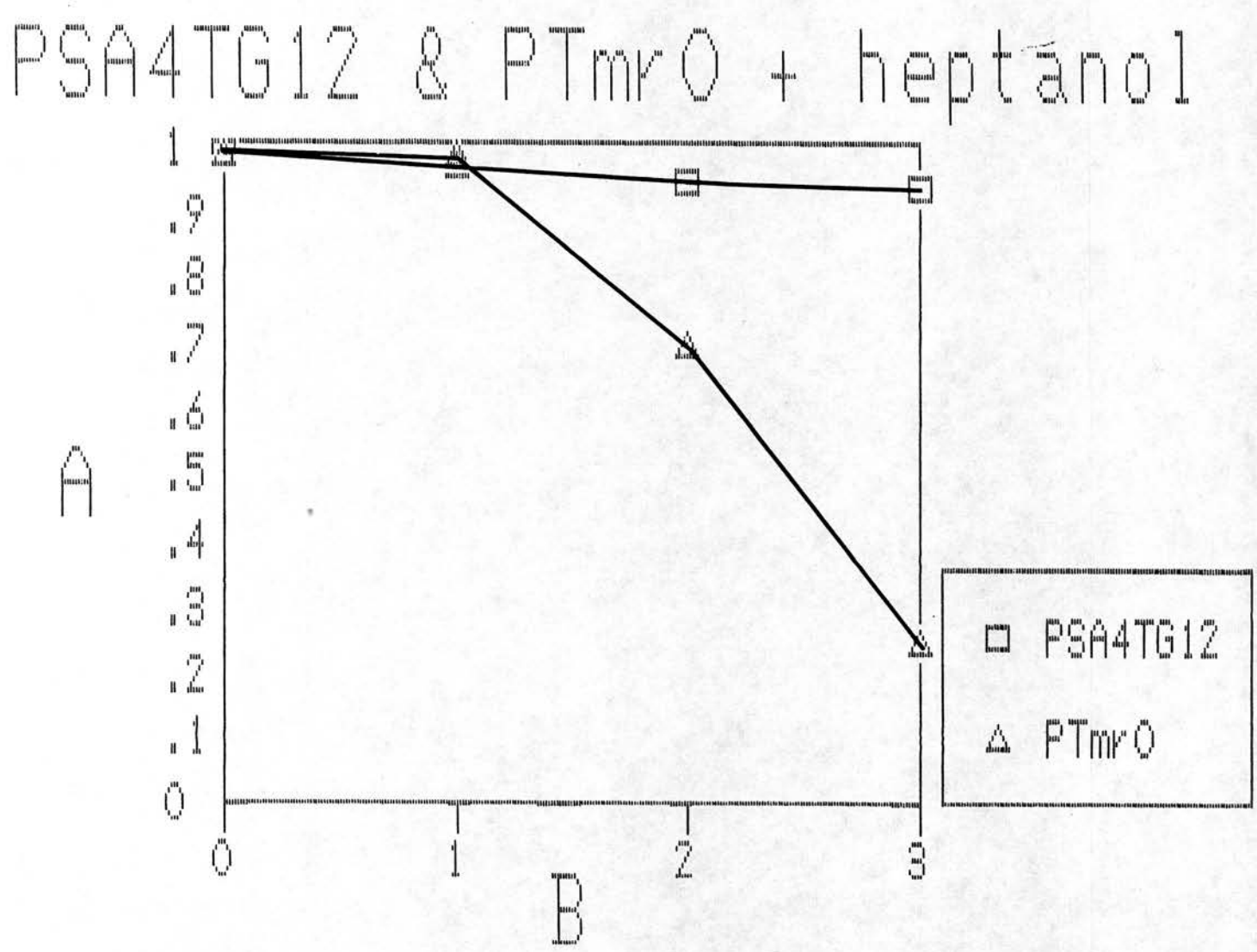

PSA4TG12 & PTmr0 + heptanol
A
B
0
1
2
3
0 = 1
= 2
= 3
= 4
= 5
= 6
= 7
= 8
= 9
PSA4TG12
PTmr0

FIGURE 5.5

Uridine nucleotide transfer indices for PSA4TG12 and PTmr0 cells incubated for 24 hours with heptanol (experiment 2).

Abscissa = concentration of heptanol (mM)

Ordinate = proportion of positive contacts

PS4TG12 & PTMPO + heptanol
PS4TG12
PTMPO
T
B
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3

Uridine nucleotide transfer analysis provides compelling evidence that these alcohols are potent inhibitors of intercellular communication in all three lines (with the exception of one anomalous result with PSA4TG12), and that in the case of B2B2TG5α, this inhibition is rapidly established and reversible. These observations are consistent with the findings of other workers concerning the effects of heptanol and octanol upon junctional communication (section 1.15).

In the light of the uridine transfer results, the high survival of cells observed in the ouabain rescue assay is somewhat surprising. There may, however, be a very straightforward explanation for this. It is possible that heptanol and octanol evaporate from the ouabain rescue plates at a rate sufficient to rapidly diminish their concentrations below a threshold level for potent inhibition of gap junction mediated communication. Given that the surface area to volume ratio of medium in 60mm plates containing 5mls of medium as standard (5.65 cm^2 per ml of medium) is higher than that for the 30mm plates used for uridine transfer and containing 2mls of medium as standard (3.53 cm^2 per ml), and that in all cases uridine transfer plates were replenished with heptanol or octanol upon seeding with donor cells so that they only had three hours during which evaporation could take place, as compared to a full twenty-four hours for ouabain rescue plates (heptanol and octanol replenished daily), this would seem to be a highly plausible

explanation. The possibility also exists that heptanol and octanol alter the pore-size of gap junctions in B2B2TG5α in such a way that they are still permissive for the passage of small cations but not for the passage of nucleotides. Considering that treatment with heptanol and octanol has been shown to strongly inhibit electrical coupling between cells in a number of other systems (Bernardini *et al*., 1982 & 1984; Johnston *et al*., 1980; Ramon *et al*., 1985), this would seem to be highly unlikely.

The results of uridine transfer analyses of the effect of heptanol and retinoic acid upon junctional communication in the metabolic cooperation variant PTmr0 and its parent line PSA4TG12 show that junctional communication in the PSA4TG12 control was inhibited by both treatments (in one experiment). However, retinoic acid at a concentration of 10^{-4}M had little effect upon junctional communication in PTmr0, while 3mM heptanol caused strong inhibition. This is an interesting result in the light of the suggestion made by Pitts *et al* (1986) that the mechanism of inhibition of junctional communication by retinoic acid might be related to that of octanol (and, by inference, to that of heptanol). Certainly, both retinoic acid and octanol seem to affect gap junction gating directly, and not by altering the rate of turnover of the gap junction protein or membrane fluidity in the vicinity of junctional plaques (section 1.15). From these results, it would appear at first sight that the mechanism of

inhibition by retinoic acid may not be the same as that of octanol. However, since the mechanism(s) in operation here have not been identified this statement should be treated with due caution. It is possible that both retinoic acid and heptanol/octanol operate indirectly via intermediate pathways which converge at some point, resulting in the closure of the junctional channel by a common mechanism. If this is the case it is conceivable that the lesion in PTmr0 may affect only the retinoic acid specific part of the pathway, and not the part relating to heptanol or octanol. It would obviously be of value to extend the scope of this investigation by selecting a battery of variant cell lines resistant to the effect of a range of different inhibitors of intercellular communication. Using the above approach it might then be possible to build up a fairly comprehensive picture of the relationship between various inhibitors of junction-mediated intercellular communication.

CHAPTER 6

A METHOD FOR THE PRODUCTION OF BRL-CONDITIONED MEDIUM USING MICROCARRIER CULTURE

6.1 INTRODUCTION

It has recently been found that medium conditioned by incubation with Buffalo Rat Liver cells (BRLcm) exerts an inhibitory effect upon the differentiation of EC and ES cells which is analogous to the feeder effect (Smith and Hooper, 1987). The standard method for producing BRLcm has been to incubate 30mls of medium upon a mono-layer of BRL cells in a 175cm² flask for three days. It is then filtered and diluted to 70% for use. Considering the large volumes of media required for my selections, and the fact that BRLcm is stable for long periods when stored frozen at -20°C, I felt that a bulk method of production, whereby several litres of BRLcm could be produced within a very short time and then frozen for long-term use, was desirable. The obvious solution was to utilise microcarrier culture, and this chapter describes the optimisation of BRL-conditioned medium production upon Cytodex-3 microcarriers. The method for their use is described in sections 2.32 and 2.35.

6.2 OPTIMISATION OF BRL-CONDITIONED MEDIUM PRODUCTION USING CYTODEX-3 MICROCARRIERS

Cytodex-3 microcarriers have a surface area of approximately 4600cm²/g (Pharmacia microcarrier culture handbook). Initially three BRL cell cultures were set up at microcarrier concentrations of 0.63g/l, 1.26g/l and

2.5g/l, which are approximately equivalent in surface area to volume ratio to 60ml medium per 175cm² flask, 30ml/flask and 15ml/flask respectively. After three days incubation, media were filtered, diluted to 70% with CMβ and used to feed three freshly inoculated cultures of B2B2TG5 in 25cm² flasks. In cultures fed with BRLcm produced at 0.63g microcarriers/l and 1.26g/l a small amount of heterogeneity was observed after three days in culture, whereas the culture fed with BRLcm produced at 2.5g/l was completely homogeneous, as was a control culture fed with BRLcm conditioned for three days at 60ml/175cm² flask. Therefore I decided to produce a quantity of BRLcm upon microcarriers at 2.5g/l and carry out plating tests at a range of dilutions to determine optimal growth conditions.

Six x 100mm tissue culture plates were each seeded with 3000 B2B2TG5 cells and fed with the test medium at dilutions of 0, 25, 40, 60, 75 and 100%, and maintained for ten days, being re-fed every other day. After this period colonies were fixed and stained with Leishmans' stain. Colony counts were performed and colony areas measured using an IBAS-2 image analyser, and the extent of differentiation was qualitatively assessed by eye. From this information, optimal growth was calculated to occur between 25% and 60% BRLcm, with the abundance of differentiated cell types diminishing with increasing BRLcm concentration (table 6.1, figure 6.1). 60% dilution was therefore adopted as standard. At 100% BRLcm there

TABLE 6.1

Microcarrier-produced BRL conditioned medium; the effect of BRLcm concentration upon growth and differentiation of B2B2TG5.

Medium conditioned upon 2.5g microcarriers/l.

% BRL	No. of colonies	Mean colony size (mm)	Total area per plate (mm^2)	Proportion of differentiated colonies
0	18	0.2729	4.9	all
25	547	0.9605	525.4	+++
40	520	0.8278	430.5	+++
60	419	0.7722	323.6	++
75	272	0.5489	149.3	+
100	66	0.4008	26.5	-

Note: +++ ≈ 90%
++ ≈ 50%
+ ≈ 20%
- = <5%

The above criteria refer to the presence of any differentiated cell types associated with cell colonies. At BRLcm concentrations above 25%, the majority of colonies scored as differentiated were composed of approximately 90% or more ES cells (by area), differentiated cell types being present only at their periphery.

FIGURE 6.1

Plot of total cell colony area vs concentration for BRL-conditioned medium produced in microcarrier culture (microcarrier density = 2.5g/l).

Abscissa = percentage (v/v) of BRL-conditioned medium in CMβ

Ordinate = total colony area per plate

Microcarrier produced BRLcm
T
B
0
10
20
30
40
50
60
70
80
90
100
0
180
280
380
480
580

were very few surviving colonies, confirming the observation of Smith (1986) that cell growth is inhibited at high concentrations of BRLcm. The plating efficiency of cells in this test was low (maximum plating efficiency = 18.2%) as compared to that of an earlier plating efficiency test performed with flask-conditioned BRLcm (plating efficiency = 31.5%, table 6.2). A second experiment was thus performed both to clarify this point and to confirm the observation that the surface area: volume ratio required for microcarrier-conditioned BRLcm to be effective was higher than that for flask-conditioned BRLcm.

For this experiment, four microcarrier cultures were set up at concentrations of 0.63, 1.26, 2.5 and 3.8g microcarriers per litre (unfortunately the 2.5g/l culture became contaminated and had to be discarded). After confluence had been achieved, two batches of test media were harvested from these cultures. The first batch was supplemented with new-born calf serum ($BRLcm_{nbcs}$), and the second batch with foetal calf serum ($BRLcm_{fcs}$) (taken from 1.26g/l and 3.8g/l cultures). B2B2TG5 cells which had been maintained in flask-conditioned BRLcm were plated at 1000 cells/60mm plate into the relevant medium and maintained for ten days, being re-fed every other day. BRLcm was diluted to 60% throughout and the plates were set up in duplicate. After the ten day incubation plates were fixed and stained with Leishmans' stain, and colonies counted and scored for the extent of

TABLE 6.2

Plating efficiency of B2B2TG5 cells in flask-conditioned BRLcm (60ml/flask) and upon STO feeder layers
All BRLcm diluted to 60%.

(mean of three determinations, initial inoculum 1000 cells per plate).

growth conditions	serum type	mean no. of colonies	plating efficiency (%)
BRL	ncs	315	31.5
BRL	fcs	199	20
STO	ncs	553.3	55
STO	fcs	497	50

ncs = newborn calf serum
fcs = foetal calf serum

differentiation. No attempt was made to calculate colony area in this experiment. These results are presented in table 6.3 and figure 6.2. Plating efficiencies in this experiment ranged from 44.7% to 64.5%. BRLcm produced at microcarrier densities of 0.63 and 1.26g/l did not inhibit differentiation efficiently. 62% and 63% of colonies respectively exhibited widespread differentiation in $BRLcm_{mcs}$, and 41% in $BRLcm_{fcs}$ (at 1.26g/l). Both $BRLcm_{mcs}$ and $BRLcm_{fcs}$ produced upon 3.8g microcarriers/l strongly inhibited differentiation, 80% and 76% of colonies respectively being totally undifferentiated and the remainder exhibiting only a small proportion of differentiated cell types at the periphery of large undifferentiated ES colonies. These results correlate well with the former tests upon microcarrier-produced BRLcm. In view of the strong inhibition of differentiation and the relatively high plating efficiency observed with 60% BRLcm produced under these conditions, 3.8g/l was adopted as the standard microcarrier density for BRLcm production.

One of the problems with producing, for example, four months supply of BRLcm within two or three weeks is that one cannot always accurately predict ones future media requirements (i.e. what proportion of BRLcm should be supplemented with new-born calf serum, and what proportion with foetal calf?). It was apparent that this dilemma would easily be solved if it were possible to condition serum-free medium which could then be

TABLE 6.3

Microcarrier-produced BRL conditioned medium; the effect of microcarrier density.

All BRLcm diluted to 60%. throughout.

(mean of two determinations, initial inoculum 1000 cells per 60mm plate).

microcarrier density (g/l)	Serum type	Mean no. of colonies	% differentiated colonies
0.63	ncs	520	62
1.26	ncs	447	63
3.8	ncs	645	20
1.26	fcs	609.5	41
3.8	fcs	662	24

ncs = newborn calf serum
fcs = foetal calf serum

Note: The heading "% differentiated colonies" refers both to fully differentiated colonies and to colonies containing some differentiated cells. In the majority of cases there was a small number of differentiated cells at the periphery of a large colony of ES cells. No attempt was made to separate these into different categories.

FIGURE 6.2

The effect of microcarrier density upon the quality of new-born calf serum or foetal calf serum supplemented BRL-conditioned medium.

(BRLcm diluted to 60% (v/v) with CMβ of the appropriate sera-type)

Abscissa = density of microcarriers in culture (g/l)

Ordinate = number of colonies per plate

N.B ncs = new-born calf serum
fcs = foetal calf serum
dark bar = total number of colonies
light bar = total number of differentiated colonies

colonies
diff
7000
6000
5000
4000
3000
2000
1000
0
0.45
1.24
3.0
1.24
3.0
FCS
FCS

supplemented with whatever type of serum was required at the time. Therefore a third differentiation test was performed to test this possibility. Microcarrier cultures were set up at densities of 0.63, 1.26, 2.5 and 3.8g/l, and three 175cm² flask cultures also set up for comparison. These were used to condition medium at 15, 30 and 60ml per flask. When cultures had reached confluence, medium supplemented with new-born calf serum was added and conditioned for three days. This was followed by serum-free medium, also for three days. This cycle was repeated three times. Immediately prior to testing, serum-free media were supplemented with 10% (v/v) new-born calf serum, and all media were then diluted to 60% with freshly prepared CMβ(ncs). Media were tested upon duplicate cultures of B2B2 cells seeded onto 60mm plates at 500 cells per plate. Plates were maintained in culture for ten days and scoring was carried out as in the previous experiment. Although plating efficiency was considerably lower and differentiation somewhat higher than in the previous experiment, media conditioned in the absence of serum were comparable with the serum-supplemented controls, and the microcarrier conditioned medium appeared to support ES cell growth slightly better than the flask conditioned medium (table 6.4 and figures 6.3 and 6.4) (in view of the small numbers of colonies involved this is possibly due to experimental variation). The poor plating efficiency of cells grown in medium conditioned at 15ml/175cm² flask is consistent with previous observations that high

TABLE 6.4

Serum-free and serum-supplemented BRL conditioned medium production using microcarriers and 175cm² flasks.

(BRLcm diluted to 60% throughout. Initial inoculum 500 B2B2 cells per 60mm plate, mean of two determinations).

microcarrier density (g/l)	mls medium per flask	serum	no. of colonies	differentiated colonies (%)
0.63	-	ncs	98	59
		-	62.5	69
1.26	-	ncs	95	64
		-	97	78
2.5	-	ncs	83.5	66
		-	114	50
3.8	-	ncs	131	43
		-	113	42
-	15	ncs	39	31
		-	65	19
-	30	ncs	86.5	57
		-	78	62
-	60	ncs	98	71
		-	77.5	67

Note: The heading "% differentiated colonies" refers both to fully differentiated colonies and to colonies containing some differentiated cells. In the majority of cases there was a small number of differentiated cells at the periphery of a large colony of ES cells. No attempt was made to separate these into different categories.

FIGURE 6.3

A comparison between BRLcm conditioned in the presence of 10% (v/v) new-born calf serum, and in the absence of serum, at various microcarrier densities.

(BRLcm diluted to 60% (v/v) with CMβ, and serum-free medium supplemented with 10% (v/v) new-born calf serum for use)

Abscissa = density of microcarriers in culture (g/l) and serum supplementation during conditioning

Ordinate = number of colonies per plate

N.B. ncs = new-born calf serum
s/f = serum-free
dark bar = total number of colonies
light bar = total number of differentiated colonies

Microcalyx leaf-produced BALCM
150
100
50
0
colonies
diff
ncs s/f
0.63
ncs
1.24
ncs s/f
2.5
ncs s/f
5.0

FIGURE 6.4

A comparison between BRLcm conditioned in the presence of 10% (v/v) new-born calf serum, and in the absence of serum, in 15, 30 and 60ml quantities in 175cm² flasks.

(BRLcm diluted to 60% (v/v) with CMβ, and serum-free medium supplemented with 10% (v/v) new-born calf serum for use)

Abscissa = volume of medium conditioned per flask (ml) and serum supplementation during conditioning

Ordinate = number of colonies per plate

N.B. ncs = new-born calf serum
s/f = serum-free
dark bar = total number of colonies
light bar = total number of differentiated colonies

Flask-produced BRLCM
colonies
diff
150
100
50
0
ncs s/f 60
ncs s/f 30
ncs s/f 15

concentrations of BRL-produced factors inhibit ES cell growth (section 1.6). Therefore it is concluded that the conditioning of serum-free medium is a viable proposition.

6.3 DISCUSSION AND SUMMARY

The inhibition of EC and ES cell differentiation by BRL-conditioned medium has proved to be of great utility, both for routine stem cell culture and for applying experimental techniques which in the past have been severely hampered by the presence of feeder cells. In my case, the use of BRLcm has been of paramount importance for the optimisation of conditions for the thioguanine kiss-of-death selections. As cell numbers in selections are necessarily high, requiring correspondingly large quantities of medium, and the bulk production of BRLcm in 175cm^2 flasks is a time-consuming process, I describe in this chapter the development of a simple method for the large-scale production of BRLcm in microcarrier culture.

It will be noted from the results presented in this chapter that for both flask-conditioned and microcarrier-conditioned medium a compromise has to be reached between the potency of differentiation inhibition and optimal growth rates. While high concentrations of BRLcm (in excess of approximately 70%) inhibit differentiation with high efficiency, there is also a

severe loss of cell survival accompanied by a reduction in growth rate. At lower concentrations (between approximately 25% and 40%), cell survival and growth rate is high but is accompanied by extensive differentiation. In fact, the results of the image analysis may be somewhat misleading as to the overall growth rate at low concentrations of BRLcm since it is feasible that the image analyser was not selective enough to accurately delineate the border between undifferentiated ES cells and the differentiated cells which typically appear at the periphery of colonies, and tend to be considerably larger than their ES cell progenitors. Thus, differentiated colonies may have occupied a greater area than undifferentiated colonies, while possessing fewer cells. The other factor which has been taken into account is the relationship between the surface area: volume ratio of BRL cells to medium and the effect which this has upon the quality of the resultant conditioned medium at a given concentration. From the single determination carried out upon flask-conditioned medium it would appear that the best compromise between plating efficiency and inhibition of differentiation was attained at around 30mls of medium per 175cm^2 flask (as differentiation in this test was generally higher than expected this should be regarded with some caution, although in mass culture there is no noticeable differentiation when medium produced under these conditions is employed). Conditioning medium at 15mls per flask resulted in a reduction in both the extent of

differentiation and in plating efficiency, in a similar manner to that observed with high concentrations of BRLcm in the initial experiment, while conditioning medium at 60mls per flask gave rise to a higher proportion of differentiated colonies than with the 30ml/flask conditioned medium.

It is immediately obvious from the results presented herein that the surface area:volume ratio required for microcarrier-conditioned BRLcm to efficiently inhibit differentiation is considerably greater than that for flask-conditioned medium. There are two likely reasons for this. First, a proportion of microcarriers do not come into contact with BRL cells during the initial inoculation procedure. Therefore the total area occupied by BRL cells is always somewhat lower than the total available surface area in the culture. Second, if stirring is too slow to maintain all occupied microcarriers in suspension, or if the inoculation procedure is over-long, large clusters of occupied microcarriers tend to form. This can be a cumulative effect, since the clumps of occupied microcarriers will tend to settle out at normal stirring speeds and aggregate with other such clumps, and with any solitary microcarriers which they may encounter. This reduces the effective surface area of BRL cells exposed to the outside environment and may result in a corresponding drop in secretion of the differentiation inhibiting activity into the culture medium. The simple expedient of

increasing stirring speed to prevent such aggregation or to break up existing aggregates is not practicable since the resultant increase in shearing forces within the culture vessel tends to cause cells to be stripped from the microcarriers. Obviously, this phenomenon places an upper limit upon the overall density of microcarriers which can be employed in a suspension system and for this reason I consider that it would be technically difficult to investigate the effects of conditioning medium at densities above 3.8g/l using our microcarrier culture system.

Despite these technical problems it has proved possible to produce BRLcm of good quality using 3.8g of microcarriers per litre, provided that the initial inoculation phase was monitored carefully. Furthermore, the finding that medium can be conditioned in the absence of serum with no significant loss in plating efficiency, or inhibition of differentiation, as compared to medium supplemented with 10% (v/v) new-born calf serum during conditioning, may prove to be valuable insofar as it will solve the logistical problem of forward planning when different sera-types are likely to be employed in the course of an investigation. BRL cells appeared to remain healthy while conditioning serum-free medium. However, serum-free medium was routinely alternated with serum-supplemented medium in a three-day cycle, and so whether a BRL cell culture could support the conditioning of serum-free medium on a long-term basis remains to be

established.

Although the proportion of differentiated colonies in test plates was in no instance below 19%, it must be pointed out that the criteria for scoring colonies as differentiated were strict, and that for the higher concentrations of microcarriers, colonies scored as being differentiated were almost exclusively composed of a large core of undifferentiated ES cells with only a small number of differentiated cell types at the periphery. Also, in mass culture BRLcm conditioned at 3.8g microcarriers/l appears to completely inhibit differentiation. Possibly in this instance the ES cells condition their own medium to some extent (Martin G.R., 1981), enhancing the effect of the BRL medium, whereas at low density insufficient ES cells are present for this to occur. All of the cell culture work presented in this thesis was carried out in microcarrier-conditioned medium produced at 3.8g/l with no apparent deleterious effect upon the differentiation capacities of cell lines, even after prolonged periods in culture.

In short, microcarrier-based production of BRLcm is a viable alternative to the standard, flask-based production method and represents a considerable saving in both time and expense (at 3.8g of microcarriers/l, and 30ml of medium/flask, microcarriers are about 6 times cheaper to use than flasks). It is hoped, therefore, that this investigation will prove to be of value to workers

in the field of stem cell biology.

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DECLARATION

I declare that this thesis is entirely my own work and has been composed by myself except where otherwise indicated.